STUDIES IN CHEMOTHERAPY

Thesis

presented by

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April 1951.

As partial fulfilment of the requirements

for the Degree of D.Sc.,

University of Edinburgh.

C O N T E N T S

STUDIES IN CHEMOTHERAPY

1. Outline of the nature of the published papers.

The papers presented in the field of chemotherapy fall into two groups:

(a) Papers on antimalarials;

(b) Papers on antibiotic peptides and amino acids.

In addition, the candidate presents, along with and as part of this thesis, his book, "The Basis of Chemotherapy", as an original contribution to the theoretical development of the subject.

2. List of papers published on chemotherapy.

1. King, H. and Work, T.S. Antiplasmodial action and chemical constitution. Part III. Carbinolamines derived from naphthalene and quinoline. J.chem.Soc., 1940, p.1307.

2. Work T.S. Antiplasmodial action and chemical constitution. Part IV. The synthesis of some complex carbinolamines and polyamines. J.chem.Soc., 1940, p.1315.

3. King, H. and Work T.S. Antiplasmodial action and chemical constituion. Part V. Carbinolamines derived from 6-methoxyquinoline. J.chem.Soc., 1942, p.401.

4. Work. T.S. Antiplasmodial action and chemical constitution. Part VI. Compounds related to lepidylamine. J.chem.Soc., 1942, p.426.

5. Work. T.S. The synthesis of amines from amides through amidodichlorides. J.chem.Soc., 1942, p.429.

6. Work, T.S. Antiplasmodial action and chemical constitution. Part VII. Derivatives of quinine and isoquinine. J.chem.Soc., 1944, p.334.

7. Work, T.S. The synthesis of antimalarial compounds related to niquidine. Part I. Model experiments on the synthesis of quinolyl carbinols. J.chem.Soc., 1946, p.194.

8. Work, T.S. The synthesis of antimalarial compounds related to niquidine. Part II. Synthesis of a dihydro-x-niquidine. J.chem.Soc., 1946, p.197.

9. Work, T.S. The synthesis of antimalarial compounds related to niquidine. Part III. Alternative synthesis of dihydro-x-niquidine. J.chem. Soc., 1947, p.222.

10. Tonkin, I.M. and Work, T.S. A new antimalarial drug. Nature, 1945, 156, 630.

11. Work, T.S. d- and l-amino acids in antibiotics. Biochem.Soc.Symposia, 1948, No. 1, p.61.

12. Harris, J.I. and Work, T.S. Synthetic pentapeptides related to gramicidin-S. Nature, 1948, 161, 804.

13. Harris, J.I. and Work, T.S. Lysine analogues as inhibitors of bacterial growth. Biochem.J., 1950, 46, 190.

14. Harris, J.I. and Work, T.S. The synthesis of peptides related to gramicidin-S and the significance of optical configuration in antibiotic peptides. I. Tri-peptides. Biochem.J., 1950, 46, 196.

15. Harris, J.I. and Work, T.S. The synthesis of peptides related to gramicidin-S and the significance of optical configuration in antibiotic peptides. II. Penta-peptides. Biochem.J., 1950, 46, 582.

16. Work, T.S. A discussion on antibiotic acitivity of growth factor analogues. Proc.Roy.Soc., B, 1949, 136, 159.

17. Work, T.S. and Work, E. The Basis of Chemotherapy. Oliver & Boyd, Edinburgh, 1948, xx, 435 p.

3. Other original work presented with this thesis.

Since 1936, when the candidate was awarded the degree of Ph.D., he has worked also in the field of natural product chemistry and has published original work on vitamin E, Cannabis indica resin, analogues of acetyl choline, the toxic factors in bleached wheat flour, and on local anaesthetics.

4. List of papers relating to 3.

18. Todd, A.R., Bergel, F., Waldmann, H. and Work, T.S. Constituents of vitamin E concentrates from rice- and wheat-germ oils. Nature, 1937, 140, 361.

19. Todd, A.R., Bergel, F., Waldmann, H. and Work, T.S. Studies on vitamin E. I. The isolation of some crystalline alcohols from the unsaponifiable matter of rice-and wheat-germ oils. Biochem.J., 1937, 31, 2247.

20. Todd, A.R., Bergel, F. and Work, T.S. Studies on vitamin E. II. The isolation of β-tocopherol from wheat-germ oil. Biochem.J., 1937, 31, 2257.

21. Bergel, F., Jacob, A., Todd, A.R., and Work, T.S. Vitamin E Structure of β-tocopherol. Nature, 1938, 141, 646.

22. Bergel, F., Todd, A.R. and Work, T.S. Studies on vitamin E. Part III. Observations on the structure of α- and β-tocopherol. J.chem. Soc., 1938, p.253.

23. Bergel, F., Jacob, A., Todd, A.R. and Work, T.S. Vitamin E synthesis of α-tocopherol. Nature, 1938, 142, 36.

24. Bergel, F., Jacob, A., Todd, A.R. and Work, T.S. Studies on vitamin E. Part IV. Synthetic experiments in the coumaran and chroman series. The structure of the tocopherols. J.chem.Soc., 1938, p.1375.

25. Bergel, F., Copping, A.M., Jacob, A., Todd, A.R. and Work, T.S. Studies on vitamin E. Part V. Synthesis of racemic α-tocopherol and of a lower homologue. J.chem.Soc., 1938, p.1382.

26. Jacob, A., Steiger, M., Todd, A.R. and Work, T.S. Studies on vitamin E. Part VI. Synthesis of lower homologues of α-tocopherol. J.chem.Soc., 1939, p.542.

27. Work, T.S., Bergel, F. and Todd, A.R. The active principles of Cannabis indica resin. I. Biochem.J., 1939, 33, 123.

28. Work, T.S. The synthesis of N-trimethylglycylcholine. J.chem.Soc., 1941, p.190.

29. MacIntosh, F.C. and Work, T.S. Some aminoethanol derivatives possessing local anaesthetic acitivty. Quart.J.Pharmacy, 1941, 14, 16.

30. Work, T.S. The biochemical effects of mutation. Ann.Rep.Chemical Society, 1947, p.254.

31. Campbell, P.N., Work, T.S. and Mellanby, Sir E. Isolation of a crystalline toxic factor from agenized wheat flour. Nature, 1950, 165, 345.

32. Campbell, P.N., Work, T.S. and Mellanby, Sir E. The isolation of a toxic substance from agenized wheat flour. Biochem. J., 1951, 48, 106

Reprinted from the Journal of the Chemical Society, 1940.

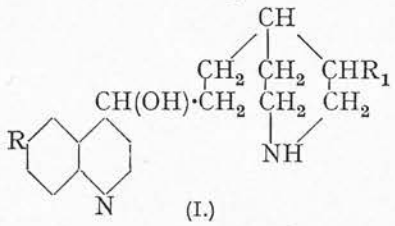
248. *Antiplasmodial Action and Chemical Constitution. Part III. Carbinolamines derived from Naphthalene and Quinoline.*

By Harold King and Thomas S. Work.

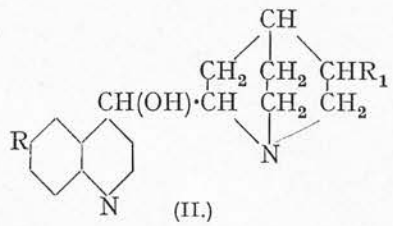
The aim of this investigation was the preparation of antiplasmodial substances based on the formula of quinine but of simpler structure. A series of 1 : 2-carbinolamines has been prepared from naphthalene and quinoline and from their methoxy-derivatives. Antiplasmodial activity has been found in the dibutyl-, diamyl-, and dihexyl-aminomethyl-6-methoxy-4-quinolylcarbinols. These are among the simplest substances to show antiplasmodial activity.

In Part II (Ainley and King, *Proc. Roy. Soc.*, 1938, *B*, **125**, 60) it was shown that, although *d*- and *l*-dihydroquinicinols, which are γ-piperidine derivatives (I; R = OMe, R_1 = Et) bore so close a resemblance to dihydroquinine (II; R = OMe, R_1 = Et), they were devoid of any significant antiplasmodial action as tested on bird malaria. When, how-

ever, the piperidine ring was attached at its α-position through the carbinol group to the methoxyquinoline nucleus as in (III; R = OMe), two diastereoisomerides were obtained, both of which showed antiplasmodial activity.

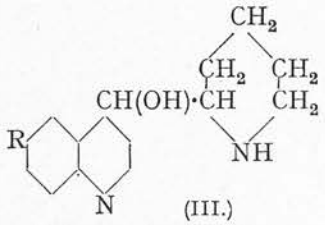


(I.)

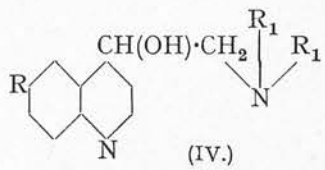


(II.)

Since the latter bases were difficult of access and modification by conversion into the tertiary bases led to loss of activity, it seemed desirable to prepare a more accessible series of simple carbinolamines (IV) in which the strongly basic nitrogen centre was still separated from the quinoline nucleus by two carbon atoms as in quinine or hydroquinine.



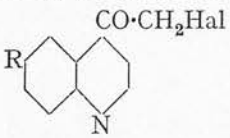
(III.)



(IV.)

A few such bases, five in all, have already been prepared by Kaufmann (*Ber.*, 1913, **46**, 1823) and by Rabe, Pasternack, and Kindler (*Ber.*, 1917, **50**, 144), but beyond the statement by Kaufmann that they are of low toxicity to humans but strongly toxic to infusoria and paramecia there is no published record of their antiplasmodial activity. Schönhöfer, however, states (*Medicine in its Chemical Aspects*, 1938, **3**, 62) that compounds prepared by Kaufmann's method were examined in the laboratories of the I.G.Farbenindustrie and found inactive on avian malaria.

For the preparation of the required carbinolamines essential intermediates were 4-quinolyl and 6-methoxy-4-quinolyl halogenomethyl ketones (V), of which the bromo-representatives were available by the method of Rabe, Pasternack, and Kindler (*loc. cit.*).



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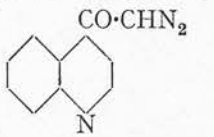
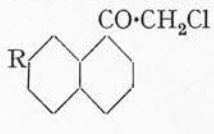


(VI.)



(VII.)

It seemed, possible, however, that the useful diazomethane reaction with acid chlorides could be applied to heterocyclic acid chlorides. In fact, the reaction between cinchoninic acid chloride and diazomethane was found to proceed normally with formation of *4-quinolyl diazomethyl ketone* (VI) and the latter readily gave *4-quinolyl chloromethyl ketone* in the usual way. The yields, however, precluded the use of this method for the preparation of large quantities of the chloromethyl ketone. Recently Baumgarten and Dornow (*Ber.*, 1940, **73**, 44; Dornow, *ibid.*, p. 185) have also applied the diazomethane reaction to other heterocyclic compounds such as the pyridinecarboxylic acid chlorides. Although the diazomethane reaction was not convenient for the reaction with cinchoninic acid, it proved quite suitable for the preparation of 1-naphthacyl chloride (VII, R = H) and 7-methoxy-1-naphthacyl chloride (VII, R = OMe) in excellent yield.

Naphthacyl chloride, 7-methoxynaphthacyl chloride, 4-quinolyl bromomethyl ketone, and 6-methoxy-4-quinolyl bromomethyl ketone were allowed to react with two molecular proportions of a series of secondary bases, usually in ethereal solution. When the separation of the secondary base hydrochloride or hydrobromide was complete, the keto-bases were straightway reduced catalytically, with palladised charcoal as catalyst, in methyl-

alcoholic solution containing aqueous mineral acid. The carbinolamines were then isolated as salts, usually with picric acid.

Representative carbinolamines have been made from a series of homologous secondary bases from dimethylamine to diheptylamine, from piperidine and from $\gamma\gamma'$-dipiperidyl. In the last-named case monosubstitution was ensured by use of the *monobenzoyl* derivative, the preparation of which was made possible by the application of the method used by Moore, Boyle, and Thorn (J., 1929, 39) for the monoacylation of piperazine.

The preparation of dihexylamine and diheptylamine presented difficulties, since they could not be made by a method analogous to the preparation of diethylamine from nitrosodiethylaniline, nor could they be obtained except in small yield by Vliet's method using sodium cyanamide (*Org. Synth., Coll. Vol.* **1**, 196). Eventually they were synthesised from *dihexyl-* and *diheptyl-benzylamine*, the benzyl group being removed by catalytic reduction with platinum oxide in glacial acetic acid at 70°.

$$CH_2Ph{\cdot}NH_2 + 2C_6H_{13}Br \longrightarrow CH_2Ph{\cdot}N(C_6H_{13})_2 \longrightarrow NH(C_6H_{13})_2$$

The removal of the *N*-benzyl group by catalytic reduction seems to have been used only (*a*) by the I.G.Farbenind. (compare B.P. 318,488, 1929, and others), where no details of the conditions or catalyst are recorded, and (*b*) by Baltzly and Buck (*J. Amer. Chem. Soc.*, 1940, **62**, 164) (compare, however, Bergmann and Zervas, *Ber.*, 1932, **65**, 1192, footnote).

The results of the tests on bird malaria due to *Plasmodium relictum* (= *Pl. præcox*) in canaries, of the carbinolamines described in this communication are shown in the following table.

Substance.	Dose in mg. per 20 g. of body weight.	Day of appearance of parasites in blood.	Remarks.
Dimethylaminomethyl-1-naphthylcarbinol	6 × 2·5 *	5	
	6 × 2·5	5	
Piperidinomethyl-1-naphthylcarbinol	6 × 1·25	5	
	6 × 1·25	5	
Piperidinomethyl-7-methoxy-1-naphthylcarbinol	6 × 0·625	5	M.T.D.†
	6 × 0·625	5	
Diethylaminomethyl-4-quinolylcarbinol	6 × 1·25	5	
	6 × 1·25	5	
Piperidinomethyl-4-quinolylcarbinol	6 × 5	5	
	6 × 5	5	
4′ : 4″-Piperidylpiperidinomethyl-4-quinolylcarbinol	6 × 5	5	M.T.D.
	6 × 5	5	
Diethylaminomethyl-6-methoxy-4-quinolylcarbinol	1 × 2·5 + 5 × 1·25	5	
	6 × 2·5	5	
Dibutylaminomethyl-6-methoxy-4-quinolylcarbinol	1 × 5 + 1 × 2·5 + 4 × 1·25	10	
	6 × 2·5	9	
	6 × 1·25	8	
Diamylaminomethyl-6-methoxy-4-quinolylcarbinol	6 × 10	10	
	6 × 10	11	
	6 × 5	5	M.T.D.
	6 × 5	9	
Di*iso*amylaminomethyl-6-methoxy-4-quinolylcarbinol	2 × 2·5 + 4 × 1·25	5	M.T.D.
	6 × 2·5	5	
Dihexylaminomethyl-6-methoxy-4-quinolylcarbinol	6 × 10	13	
	1 × 10 + 5 × 5	12	
	6 × 5	10	M.T.D.
	6 × 5	7	
Diheptylaminomethyl-6-methoxy-4-quinolylcarbinol	6 × 5	5	M.T.D.
	6 × 5	5	
Piperidinomethyl-6-methoxy-4-quinolylcarbinol	6 × 5	6	
	6 × 5	5	
4′ : 4″-Piperidylpiperidinomethyl-6-methoxy-4-quinolylcarbinol	6 × 5	6	M.T.D.
	6 × 5	5	
	6 × 2·5	5	
	6 × 2·5	6	
Quinine	6 × 2·5	12—14	
Control birds	—	5	

* This means a dose of 2·5 mg. was given daily for 6 days, the first dose being administered 4 hours after inoculation with malaria.

† M.T.D. = maximum tolerated dose.

A perusal of this table shows that no naphthyl, methoxynaphthyl, or quinolyl derivative had any noticeable activity. However, in the methoxyquinolyl series there was a zone of activity, the three bases dibutyl-, diamyl-, and dihexyl-aminomethyl-6-methoxy-4-quinolylcarbinols (IV) all being active, whereas the lower and the higher homologues were inactive. It is of interest that the dibutyl compound, for instance, differs in its molecular formula from dihydroquinine by having four extra hydrogen atoms.

Experimental.

α-*Naphthoic Acid Derivatives.*

α-*Naphthoyldiazomethane.*—Naphthoyl chloride (4·4 g.) in ether (25 c.c.) was run slowly into a cold solution of diazomethane in ether (from 10 g. of nitrosomethylurea). After being kept for 12 hours at room temperature, the ether was removed in a vacuum without heating, and the residue triturated with light petroleum. The pale yellow, crystalline product (3·5 g.), m. p. 56°, was sparingly soluble in petrol and readily soluble in benzene. A second crop of less pure material (0·9 g.) was obtained on concentrating the petroleum mother-liquor (Found: C, 73·6; H, 4·3; N, 14·3. $C_{12}H_8ON_2$ requires C, 73·5; H, 4·6; N, 14·3%).*

α-*Naphthacyl Chloride.*—Naphthoyldiazomethane (3·5 g.) was dissolved in ether, and dry hydrogen chloride passed into the solution until no more nitrogen was evolved; the ether was evaporated, and the oily product purified by distillation; yield, 94%.

Piperidinomethyl-1-*naphthylcarbinol.*—Naphthacyl chloride (5·15 g.), dissolved in ether (10 c.c.), was run slowly at room temperature into a solution of piperidine (4·6 g.) in ether (10 c.c.). Addition took about 10 minutes and the temperature rose to about 35°. After being kept for 1 hour, the precipitated piperidine hydrochloride (2·8 g.) was collected, and the filtrate evaporated in a vacuum at room temperature. No attempt was made to isolate the keto-amine, which was immediately dissolved in methyl alcohol, made acid to Congo by 3N-hydrochloric acid, and reduced catalytically with palladised charcoal as catalyst. Absorption ceased when 380 c.c. of hydrogen had been absorbed. The catalyst was removed, and the filtrate concentrated to a small volume; the desired hydroxy-amine *hydrochloride* (3·05 g.), sparingly soluble in water, then crystallised. A further crop (0·65 g.) was obtained from the mother-liquor. Crystallised from methyl alcohol, the pure product melted at 270° (Found: C, 70·4; H, 6·8; N, 4·8. $C_{17}H_{21}ON,HCl$ requires C, 70·0; H, 7·0; N, 4·8%).

Dimethylaminomethyl-1-*naphthylcarbinol.*—The method was the same as in the preceding experiment. Naphthacyl chloride (2·3 g.) condensed with dimethylamine (8 c.c. 33% solution) gave 2·1 g. of oily hydroxy-amine after reduction. The base gave a crystalline *picrate* sparingly soluble in methyl alcohol, m. p. 178—180° (Found: C, 54·0; H, 4·7; N, 13·1. $C_{14}H_{17}ON,C_6H_3O_7N_3$ requires C, 54·1; H, 4·5; N, 12·6%).

Diethylaminomethyl-1-*naphthylcarbinol.*—Naphthacyl chloride was condensed with diethylamine in ether. After reduction the hydroxy-amine was crystallised from alcohol as the *picrate*, m. p. 136°; yield, 60% (Found: C, 56·4; H, 4·9; N, 12·0. $C_{16}H_{21}ON,C_6H_3O_7N_3$ requires C, 55·9; H, 5·1; N, 11·9%).

Diethanolaminomethyl-1-*naphthylcarbinol.*—Naphthacyl chloride and diethanolamine were condensed in methyl alcohol and after catalytic reduction the desired product was isolated by prolonged fractional crystallisation of the *picrate*, m. p. 127—128°, from benzene and from methyl alcohol; yield, 20% (Found: C, 52·3; H, 4·7; N, 10·7. $C_{16}H_{21}O_3N,C_6H_3O_7N_3$ requires C, 52·3; H, 4·8; N, 11·1%).

Dipropylaminomethyl-1-*naphthylcarbinol.*—This was isolated with difficulty as the *picrate* in 25% yield, m. p. 149—150°, from methyl alcohol (Found: C, 57·8; H, 5·7; N, 11·2. $C_{18}H_{25}ON,C_6H_3O_7N_3$ requires C, 57·6; H, 5·6; N, 11·2%).

1-*Cyanonaphthalene*-7-*sulphonic Acid.*—1-Aminonaphthalene-7-sulphonic acid (23 g.) was diazotised as described by Royle and Schedler (*J.*, 1923, **123**, 1643). The diazonium chloride so obtained was made into a thick paste with water and added slowly to a solution of cuprous cyanide, prepared by adding potassium cyanide (29 g. in 80 c.c. of water) to copper sulphate (27 g. in 100 c.c. of water), the temperature being maintained at 60—70° throughout. Addition took ½ hour. The temperature was kept at 60° for a further ½ hour, sodium chloride (60 g.) then added, and the mixture kept in the ice-chest overnight. The cyanonaphthalenesulphonic acid was collected and purified by solution in hydrochloric acid, filtration, and reprecipitation by addition of potassium chloride (90 g.); yield, 78%.

* All analyses are micro.

7-*Sulpho*-1-*naphthoic Acid.*—The cyano-sulphonic acid was hydrolysed as described by Royle and Schedler (*loc. cit.*). The yield was almost quantitative. The product melted at 335°.

7-*Hydroxy*-1-*naphthoic Acid.*—The sulphonic acid was fused with potassium hydroxide as described by Royle and Schedler (*loc. cit.*); yield, 80%.

7-*Methoxy*-1-*naphthoic Acid.*—7-Hydroxy-1-naphthoic acid (36 g.) was dissolved in aqueous sodium hydroxide (7·6 g. in 80 c.c. of water) and heated to 90° in a flask fitted with a stirrer and two dropping-funnels, one containing methyl sulphate (48 g.) and the other sodium hydroxide (22·7 g. in 120 c.c. of water). About 20 c.c. of sodium hydroxide were run in first and then sodium hydroxide and methyl sulphate simultaneously so that both were added in about 45 minutes. The solution was maintained at 90° for a further 15 minutes, cooled, washed with ether, and made acid by addition of concentrated hydrochloric acid. The white precipitate was collected and recrystallised from alcohol. The first crop melted at 165—167° (26·5 g.) and the second at 180—220°. The second crop was partially methylated and was used again in subsequent preparations. Dziewonski, Galitzerowna, and Kocwa (*Bull. Acad. Polonaise*, 1926, *A*, 209) give m. p. 167·5°.

Ethyl 7-*Methoxy*-1-*naphthoate.*—Methoxynaphthoic acid was esterified by refluxing with alcohol and concentrated sulphuric acid for 5 hours; b. p. 157—160°/0·9 mm.

7-*Methoxy*-1-*naphthoyl Chloride.*—Methoxynaphthoic acid (62·0 g.) was treated with phosphorus pentachloride (64·0 g.) in benzene, the phosphorus oxychloride and the benzene distilled off, and the white solid residue distilled at 150°/0·9 mm.; m. p. 74—76°, yield 95%.

7-*Methoxy*-1-*naphthacyl Bromide.*—The method of preparation was the same as that used for naphthacyl chloride. The acid chloride (18·0 g.) was treated with excess of diazomethane, and dry hydrogen bromide passed into the solution. The *product* was purified with difficulty by distillation, as at the pressure used there was appreciable decomposition; b. p. 165—170°/1 mm., yield 20·4 g. (Found: C, 55·7; H, 4·0. $C_{13}H_{11}O_2Br$ requires C, 55·9; H, 3·9%).

7-Methoxy-1-naphthacyl chloride was prepared similarly and had b. p. 155—160°/1 mm.

Piperidinomethyl-7-*methoxy*-1-*naphthylcarbinol.*—Methoxynaphthacyl bromide (3·0 g.) in ether was added to piperidine (1·93 g.) in ether (15 c.c.). After 1 hour the solution was warmed at 35° for 10 minutes, and the piperidine hydrobromide (1·6 g.) collected. The oil left on removing the ether was reduced at once in methyl alcohol–hydrochloric acid solution with a palladised charcoal catalyst, the consumption of hydrogen being 250 c.c. The basic fraction of the condensation (2·7 g.) was crystallised from methyl alcohol–acetone as the *hydrochloride*, m. p. 225—227° (Found: C, 67·4; H, 7·0. $C_{18}H_{23}O_2N$,HCl requires C, 67·2; H, 7·4%).

Cinchoninic Acid Derivatives.

4-*Quinolyl Diazomethyl Ketone.*—Cinchoninic acid chloride (4 g.), prepared by Späth's method (*Ber.*, 1926, **59**, 1484), in dry ether (30 c.c.) was added slowly to a solution of diazomethane (from 10 g. of nitrosomethylurea) in ether (50 c.c.). After 24 hours, the ether was removed by a current of dry air, and the residue triturated with dry ether containing 10% of its volume of benzene. The solid was crystallised from ether; yield 2·5 g., m. p. 80—81°. One more crystallisation gave the pure 4-*quinolyl diazomethyl ketone*, m. p. 83—84° (Found: C, 67·3; H, 3·9; N, 21·3. $C_{11}H_7ON_3$ requires C, 67·0; H, 3·6; N, 21·3%).

4-*Quinolyl Chloromethyl Ketone.*—The foregoing diazomethyl ketone (2·5 g.) was dissolved in ether (50 c.c.), and dry hydrogen chloride passed into the vigorously stirred solution. When nitrogen ceased to be evolved, the ethereal solution was washed with aqueous sodium carbonate, dried, and evaporated. The yellow solid was crystallised from benzene; yield 1·25 g., m. p. 101° (Found: C, 64·4; H, 4·0; Cl, 17·5. $C_{11}H_8ONCl$ requires C, 64·2; H, 3·9; Cl, 17·2%).

Ethyl 4-*Quinoloylacetate.*—The following method was found superior to that given by Rabe and Pasternack (*Ber.*, 1913, **46**, 1033). To a mixture of ethyl cinchoninate (40·2 g.) and ethyl acetate (20·0 g.), both dried over phosphoric oxide, sodamide (10 g.), powdered under benzene (60 c.c.), was added, and the mixture heated on the water-bath under reflux for 13 hours. The product was poured into water, and a solid (2·0 g.), which proved to be cinchoninic acid amide, removed. The filtrate was diluted with ether, and unchanged ethyl cinchoninate (5·5 g.) recovered from the ether–benzene solution. The alkaline aqueous solution was cooled in ice, made acid to Congo-paper and then treated with sodium bicarbonate until no more oil separated. The oil was collected and purified by precipitation of the acid sulphate from 25% sulphuric acid solution; yield 29—32 g. of base.

4-*Quinolyl Bromomethyl Ketone.*—This ketone was prepared from the preceding ester as described by Rabe, Pasternack, and Kindler (*Ber.*, 1917, **50**, 152). The hydrobromide melted at 225—227° (decomp.), the base at 75·5°.

Piperidinomethyl-4-quinolylcarbinol.—The following general method for the preparation of these basic carbinols was found more convenient than that described by Rabe *et al.* (*loc. cit.*). Powdered quinolyl bromomethyl ketone hydrobromide (2·5 g.) was added in portions during 10 minutes to a solution of piperidine (2·0 g.) in dry ether (15 c.c.), the mixture being shaken after each addition. After keeping for 1 hour at room temperature, the piperidine hydrobromide was collected, and the ether quickly removed without heating in a vacuum. The residue was dissolved in methyl alcohol (50 c.c.) and 3N-hydrochloric acid (25 c.c.) and reduced catalytically with palladised charcoal (0·25 g.). When hydrogen absorption (210 c.c.) was complete, the catalyst was collected, the methyl alcohol removed, and the aqueous liquor made alkaline. The ether-soluble oil which separated was purified through the dipicrate, m. p. 168° (decomp.). Rabe *et al.* gave m. p. 172—174° (decomp.) (Found : C, 47·4; H, 4·1; N, 14·4. Calc. : C, 47·1; H, 3·7; N, 14·3%). The hydrochloride had m. p. 160°. The yield in this instance was almost quantitative.

Diethylaminomethyl-4-quinolylcarbinol.—Powdered quinolyl bromomethyl ketone hydrobromide (5·9 g.), condensed with diethylamine (4·2 g.) in ether in the same way as is described above, gave diethylamine hydrobromide (4·5 g.) and a crystalline base, which was reduced catalytically (500 c.c. of hydrogen). The required product was isolated as the *dipicrate* (4·7 g.), m. p. 168° (Found : C, 46·5; H, 3·7. $C_{15}H_{20}ON_2,2C_6H_3O_7N_3$ requires C, 46·1; H, 3·7%). The hydrochloride, crystallised from alcohol, had m. p. 182°.

Dipropylaminomethyl-4-quinolylcarbinol.—The powdered bromo-ketone hydrobromide (6·2 g.) was added slowly to dipropylamine (5·3 g.) in ether (10 c.c.) and shaken vigorously after each addition. The temperature was maintained at 40° for 90 minutes, and the dipropylamine hydrobromide (5·4 g.) collected. The oil left on removal of the ether was reduced catalytically (460 c.c. of hydrogen) in methyl alcohol–hydrochloric acid solution. The required carbinol was crystallised from ethyl alcohol–acetone as the *dipicrate*, m. p. 153° (Found : C, 47·7; H, 4·3; N, 14·8. $C_{17}H_{24}ON_2,2C_6H_3O_7N_3$ requires C, 47·7; H, 4·1; N, 15·3%).

Diamylaminomethyl-4-quinolylcarbinol.—In this case the powdered bromo-ketone hydrobromide (5·0 g.) was added to diamylamine (7·43 g.) in acetone (10 c.c.). After being shaken for 1 hour at room temperature, the mixture was warmed at 50° for another hour, then cooled and diluted with dry ether, and the diamylamine hydrobromide (6·0 g.) collected. The solvent was removed at the ordinary temperature, and the residual oil dissolved in methyl alcohol–hydrochloric acid and reduced catalytically (340 c.c. of hydrogen). The required base was isolated with difficulty as the *dipicrate*, m. p. 142°, after repeated crystallisation from methyl alcohol–acetone; yield, 1·1 g. (Found : C, 50·0; H, 5·0; N, 14·7. $C_{21}H_{32}ON_2,2C_6H_3O_7N_3$ requires C, 50·4; H, 4·9; N, 14·3%).

N-*Benzoyl-4 : 4′-dipiperidyl.*—The monobenzoylation of dipiperidyl is a difficult process, but it can be effected by applying the method of Moore, Boyle, and Thorn (J., 1929, 39). After numerous trials the following method gave the best results. 4 : 4′-Dipiperidyl (22 g.) was dissolved in water (140 c.c.) and neutralised to bromphenol-blue with 3N-hydrochloric acid, a volume of acetone equal to the total volume of water present being then added. The solution was kept mechanically stirred at 50°, and benzoyl chloride (25 g.) run in drop by drop. The p_H of the solution was simultaneously adjusted to 3·8, *i.e.*, reddish-purple, bromphenol-blue being used as internal indicator, by running in saturated aqueous sodium acetate solution. When the addition of benzoyl chloride was complete, the acetone was removed by distillation under reduced pressure, and concentrated hydrochloric acid added. The mixture of benzoic acid and *dibenzoyl-4 : 4′-dipiperidyl* was taken up in the chloroform which had been used to extract the acidic filtrate. The chloroform solution was extracted with aqueous alkali to remove benzoic acid and then concentrated to 30 c.c. and mixed with an equal volume of ether; dibenzoyl-4 : 4′-dipiperidyl (16·5 g.) crystallised, m. p. 167° (Found : C, 76·4; H, 7·4. $C_{24}H_{28}O_2N_2$ requires C, 76·6; H, 7·5%).

The acidic aqueous mother-liquor was treated with aqueous potassium hydroxide so long as any oil separated, rise of temperature being avoided. The oil was taken up in chloroform, the mother-liquor being thoroughly extracted with more chloroform. On removal of the chloroform the residual oil was triturated with ether; the monobenzoate then went into solution, leaving 4 : 4′-dipiperidyl undissolved (5·4 g.). The monobenzoate (15·5 g.) was purified by crystallisation from acetone–methyl alcohol as the hydrobromide, m. p. 233°, or from methyl alcohol as the *perchlorate*, m. p. 268° (Found : C, 55·0; H, 6·5; N, 7·9. $C_{17}H_{24}ON_2,HClO_4$ requires C, 54·7; H, 6·7; N, 7·5%).

4′ : 4″-*Piperidylpiperidinomethyl-4-quinolylcarbinol.*—Powdered 4-quinolyl bromomethyl ketone (3·65 g.) was added slowly with shaking to monobenzoyl-4 : 4′-dipiperidyl (9·5 g.) in

acetone (20 c.c.), and the mixture kept for 2 hours. The monobenzoyldipiperidyl hydrobromide was collected, and the acetone removed. The residual oil was reduced in methyl alcohol–hydrochloric acid solution palladised charcoal being used as catalyst (240 c.c. of hydrogen). After removal of the catalyst and solvent, the free base (2·9 g.) was extracted from alkaline solution and hydrolysed by heating with constant-boiling hydrochloric acid for 48 hours on the boiling water-bath. The solution was made alkaline, and the base extracted with a large volume of ether, since it was sparingly soluble in this solvent. The yellow oil obtained on removal of the ether gave a crystalline trihydrochloride (2·1 g.), m. p. above 300° (decomp.), from alcoholic solution, and a crystalline *tripicrate*, m. p. 195° (Found: C, 45·9; H, 3·7; N, 15·5. $C_{21}H_{29}ON_3,3C_6H_3O_7N_3$ requires C, 45·6; H, 3·7; N, 16·3%).

Quininic Acid Derivatives.

6-*Methoxy*-4-*quinolyl Bromomethyl Ketone.*—Ethyl quininate (21·7 g.) and ethyl acetate (9·0 g.) were condensed in the presence of sodamide (5·0 g.) as described in the corresponding preparation from ethyl cinchoninate. The resulting β-keto-ester (14·7 g.) was converted into the bromo-ketone named above by the method of Rabe, Pasternack, and Kindler (*loc. cit.*).

Piperidinomethyl-6-*methoxy*-4-*quinolylcarbinol.*—The method of preparation was the same as is described above for the compound without the methoxy-group. The carbinol base was isolated in almost quantitative yield (Rabe *et al.* give a 75% yield). The *hydrochloride* was crystallised from alcohol–acetone; m. p. 164° (Found: C, 62·9; H, 7·4; N, 9·0. $C_{17}H_{22}O_2N_2,HCl$ requires C, 63·2; H, 7·2; N, 8·7%).

Diethylaminomethyl-6-*methoxy*-4-*quinolylcarbinol.*—Prepared in the same way as the compound without the methoxy-group, this carbinol was isolated as the *dihydrochloride*, m. p. 182—183°; yield, 48% (Found: C, 55·7; H, 7·4; N, 8·3. $C_{16}H_{22}O_2N_2,2HCl$ requires C, 55·3; H, 6·9; N, 8·1%).

Dibutylaminomethyl-6-*methoxy*-4-*quinolylcarbinol.*—The condensation of the bromo-ketone hydrobromide (5·5 g.) with dibutylamine (6·0 g.) was carried out in the same way as is described above for diamylaminomethyl-4-quinolylcarbinol. The resultant oily keto-base (3·64 g.) required 460 c.c. of hydrogen for reduction to the carbinol. The required product was isolated with difficulty as the crystalline dihydrochloride, m. p. 142°, from methyl alcohol–acetone and gave a *dipicrate*, m. p. 169°, from ethyl alcohol–acetone (Found: C, 49·2; H, 4·6. $C_{20}H_{30}O_2N_2,2C_6H_3O_7N_3$ requires C, 48·8; H, 4·6%).

When the reaction was carried out with di*iso*butylamine, di*iso*butylamine hydrobromide separated as usual. The residual base was catalytically reduced. All attempts to isolate the di*iso*butylaminomethoxyquinolylcarbinol were unsuccessful. The only product which could be isolated crystalline gave analytical figures agreeing with *methyl*-6-*methoxy*-4-*quinolylcarbinol hydrochloride*, m. p. 217° (from ethyl alcohol) (Found: C, 59·9; H, 5·9. $C_{12}H_{13}O_2N,HCl$ requires C, 60·1; H, 5·8%).

Diamylaminomethyl-6-*methoxy*-4-*quinolylcarbinol.*—The bromo-ketone hydrobromide (5·0 g.), condensed with diamylamine (6·7 g.), gave diamylamine hydrobromide (6·3 g.) and an oil requiring 300 c.c. of hydrogen for reduction. The required base was difficult to isolate, but was eventually obtained in the most strongly basic fraction when the bases were fractionated by the method of differing basicities (King, J., 1919, **117**, 991; 1935, 1381; 1936, 1276; 1939, 1157). It was crystallised as the *dipicrate* (0·9 g.), m. p. 155°, from methyl alcohol (Found: C, 49·9; H, 5·0; N, 13·8. $C_{22}H_{34}O_2N_2,2C_6H_3O_7N_3$ requires C, 50·0; H, 4·9; N, 13·7%).

*Di*isoamylaminomethyl-6-*methoxy*-4-*quinolylcarbinol.*—The bromo-ketone hydrobromide (5·5 g.), condensed with di*iso*amylamine (7·06 g.), gave di*iso*amylamine hydrobromide (6·8 g.) and an oil which required 380 c.c. of hydrogen on reduction. The required base was isolated as the *dipicrate*, m. p. 156°, from methyl alcohol, after separation into fractions of different basicities (Found: C, 49·7; H, 4·8; N, 13·7. $C_{22}H_{34}O_2N_2,2C_6H_3O_7N_3$ requires C, 50·0; H, 4·9; N, 13·7%).

Di-n-*hexylbenzylamine.*—Technical hexyl alcohol contains unsaturated substances. These were conveniently removed from the hexyl bromide prepared by the hydrobromic–sulphuric acid method (*Organic Syntheses*, Coll. Vol., I, 26) by shaking with small quantities of sulphuric acid until the extracts were practically colourless. Molecular quantities of benzylamine (21·4 g.), hexyl bromide (66 g.), and potassium hydroxide pellets (26·4 g.) were refluxed at 150° for 8 hours. The oil obtained on dilution with water was fractionally distilled at 14 mm. and gave (1) 9·3 g., b. p. to 100°, (2) 14·2 g., b. p. 140—170°, and (3) 33·0 g., b. p. 170—186°. On careful refractionation 7·4 g., b. p. 146—148°/14 mm., of n-*hexylbenzylamine* (Found: C, 81·3; H, 11·0; N, 7·5. $C_{13}H_{21}N$ requires C, 81·6; H, 11·1; N, 7·3%), and 29·7 g. of *di*-n-

hexylbenzylamine, b. p. 185°/14 mm. (Found: C, 83·1; H, 11·9; N, 5·2. $C_{19}H_{33}N$ requires C, 82·8; H, 12·1; N, 5·1%), were obtained. The latter does not form a hydrochloride, but n-*hexylbenzylamine hydrochloride* crystallises from acetone in woolly needles, m. p. 217—218° (Found: C, 68·4; H, 9·2; N, 6·0. $C_{13}H_{21}N,HCl$ requires C, 68·5; H, 9·7; N, 6·1%).

Di-n-*hexylamine*.—Di-*n*-hexylbenzylamine (27·0 g.) in glacial acetic acid (30 c.c.) was reduced at 70° in presence of 0·4 g. of freshly prepared platinum oxide for 6 hours. The filtered solution was made strongly alkaline and extracted with ether, and the ethereal solution dried over potassium hydroxide pellets. The residue left on removal of the ether was fractionated under reduced pressure, a first fraction containing toluene being removed up to a temperature of 110°/14 mm., followed by an almost quantitative yield of di-*n*-hexylamine, b. p. 122°/15 mm. (Found by Kjeldahl: N, 7·4. Calc.: N, 7·6%). If the drying with potash was omitted, a hydrated base was obtained, b. p. 114—116°/14 mm., which almost completely crystallised on keeping and corresponded to a *tetrahydrate* (Found by Kjeldahl: N, 5·5. $C_{12}H_{27}N,4H_2O$ requires N, 5·2%). The *hydrochloride*, prepared from either form of the base by solution in a little alcohol and addition of concentrated hydrochloric acid, crystallised from acetone in pearly scales, m. p. 270° (Found: C, 65·3; H, 12·4; N, 6·3. $C_{12}H_{27}N,HCl$ requires C, 65·0; H, 12·7; N, 6·3%).

Di-n-*hexylaminomethyl*-6-*methoxy*-4-*quinolylcarbinol*.—The reaction between dihexylamine (3·0 g.) and the bromo-ketone hydrobromide (1·8 g.) was carried out in the usual way, but it was necessary to heat to 60° to complete the reaction. Dihexylamine hydrobromide (2·2 g.) was recovered and the basic condensation product was reduced, with Adams's catalyst, in ethyl alcohol after neutralisation with hydrochloric acid, the hydrogen consumption being 160 c.c. The basic carbinol (1·2 g.) was purified by fractional crystallisation as the *dipicrate*, m. p. 173°, from ethyl alcohol (Found: C, 51·2; H, 5·2; N, 13·3. $C_{24}H_{38}O_2N_2,2C_6H_3O_7N_3$ requires C, 51·5; H, 5·5; N, 13·2%).

Di-n-*heptylbenzylamine*.—*n*-Heptyl bromide (16·3 g.; 1 mol.), benzylamine (5·0 g.; 1 mol.), and potassium hydroxide pellets (6·15 g.; 1 mol.) were mixed and heated in an oil-bath. There was a vigorous reaction at 150°; this temperature was maintained for 8 hours. When cold, the product was diluted with water, and the oily layer separated and fractionated. The first fraction distilled between 160° and 180°/14 mm. but mainly at 167°; yield, 2·6 g. This proved to be heptylbenzylamine. It was converted in aqueous alcoholic solution into the *hydrochloride*, which crystallised in woolly needles, m. p. 196° (Found: C, 69·1; H, 9·5; N, 6·1. $C_{14}H_{23}N,HCl$ requires C, 69·5; H, 10·1; N, 5·8%). The second main fraction, *di*-n-*heptylbenzylamine* (9·35 g.), boiled at 205°/16 mm. and showed the same b. p. on redistillation before analysis (Found: C, 83·1; H, 12·3; N, 4·6. $C_{21}H_{37}N$ requires C, 83·2; H, 12·0; N, 4·7%).

Di-n-*heptylamine*.—Diheptylbenzylamine (6·0 g.) in glacial acetic acid (10 c.c.) was catalytically reduced in presence of platinum (Adams's catalyst) at 70° approx. Reduction was complete in 6 hours. The filtered solution was made alkaline and extracted with ether, and the ethereal layer dried over potassium hydroxide. The residue after removal of the ether was fractionally distilled, the first fraction up to 120°/15 mm. containing some toluene. The main fraction, di-*n*-heptylamine, boiled between 120° and 150°/15 mm. and on redistillation had b. p. 147—148°/15 mm. The presence of water lowers the b. p. (Found by Kjeldahl: N, 6·4. Calc.: N, 6·6%). This anhydrous form has m. p. 1°, but readily becomes hydrated and then has m. p. 32—33° (Found by Kjeldahl: N, 5·1. $C_{14}H_{31}N,3H_2O$ requires N, 5·2%). When the hydrated form is kept in a desiccator over calcium chloride, it liquefies, but the crystalline trihydrate is instantly formed on treatment with water. Sabatier and Mailhe (*Ann. Chim. Phys.*, 1909, **16**, 70) and Skita and Keil (*Ber.*, 1928, **61**, 1452), however, give m. p. 30° for the anhydrous base. Sabatier and Mailhe describe the hydrochloride as hygroscopic, but this is incorrect, as was also shown by Skita and Keil. The hydrochloride crystallises in woolly needles, m. p. 255°; Skita and Keil give 250° (Found: C, 67·1; H, 12·2; N, 5·8. Calc.: C, 67·3; H, 12·9; N, 5·6%).

Di-n-*heptylaminomethyl*-6-*methoxy*-4-*quinolylcarbinol*.—The condensation of diheptylamine (12·8 g.) and the bromo-ketone hydrobromide (7·0 g.) was carried out as in the preceding experiment. Diheptylamine hydrobromide (11·0 g.) was recovered and during catalytic hydrogenation 610 c.c. of hydrogen were consumed. The acid-soluble portion of the product was fractionated by the method of differing basicities. From the most basic fraction, methyl-6-methoxy-4-quinolylcarbinol, m. p. 118°, was isolated (Found: C, 70·8; H, 6·2; N, 6·6. Calc.: C, 70·9; H, 6·5; N, 6·9%). Kaufmann, Kunkler, and Peyer (*Ber.*, 1913, **46**, 57) give m. p. 120—121°. The picrate had m. p. 183° and the hydrochloride 217°. From a slightly less basic fraction, di-*n*-heptylaminomethyl-6-methoxy-4-quinolylcarbinol (0·75 g.) was isolated as the

dipicrate, m. p. 130°, from ethyl alcohol (Found : C, 52·1 ; H, 5·4 ; N, 13·1. $C_{26}H_{42}O_2N_2,2C_6H_3O_7N_3$ requires C, 52·3 ; H, 5·6 ; N, 12·8%).

4′ : 4″ - *Piperidylpiperidinomethyl* - 6 - *methoxy* - 4 - *quinolylcarbinol*.—Monobenzoyldipiperidyl (10·4 g.) and the appropriate bromo-ketone hydrobromide (4·4 g.) combined to give an oily base (6·0 g.) requiring 370 c.c. of hydrogen for reduction. After hydrolysis with concentrated hydrochloric acid, the benzoic acid was removed, the solution made alkaline, and the required base extracted with ether. The product crystallised from aqueous acetone as the hydrated *trihydrochloride*, which was difficult to purify and decomposed above 300° without melting (Found : C, 50·1 ; H, 7·5 ; N, 8·3. $C_{22}H_{31}O_2N_3,3HCl,2H_2O$ requires C, 51·3 ; H, 7·4 ; N, 8·1%. Found for salt dried at 100° : C, 54·3 ; H, 7·3. $C_{22}H_{31}O_2N_3,3HCl$ requires C, 55·1 ; H, 6·5%).

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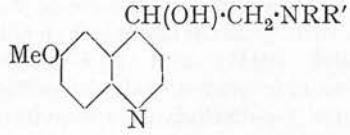
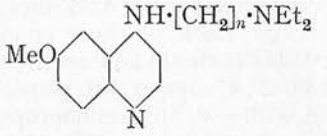
249. *Antiplasmodial Action and Chemical Constitution. Part IV. The Synthesis of some Complex Carbinolamines and Polyamines.*

By Thomas S. Work.

It has already been shown (King and Work, preceding paper) that antimalarial activity is not completely dependent on the nature of the " quinuclidine half " of compounds related to quinine. A wide variety of carbinolamines and polyamines without a quinoline nucleus have now been prepared in an attempt to assess the importance of the " quinoline half." None of the compounds prepared showed any antiplasmodial activity when tested on *Plasmodium relictum* in canaries. These results bring out the importance of the quinoline nucleus.

In Part III (*loc. cit.*) it was shown that compounds of the type (I) possessed weak antiplasmodial properties when R = R′ = butyl, amyl, or hexyl. Magidson and Rubtzow (*J. Gen. Chem., U.S.S.R.*, 1937, **17**, 1896) have also shown that weak antiplasmodial activity was shown by substances of the type (II). It is perhaps significant that bases

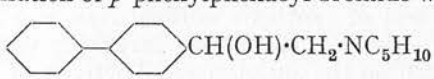
(I.) 

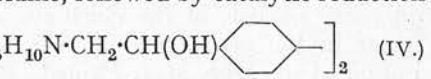


(II.)

showing antiplasmodial action, such as the cinchona alkaloids, the synthetic drugs plasmochin, atebrin, and numerous allied bases (Fourneau, *Ann. Inst. Pasteur*, 1933, **50**, 731; Magidson, *Arch. Pharm.*, 1934, **272**, 74), and substances such as (I) and (II), all have molecular weights between 300 and 400 and that structural specificity is not great, provided the molecular weight is above a certain limiting value. The aim of the present investigation was, therefore, to see whether the methoxyquinoline portion of the molecule of substances of types (I) and (II) could be replaced by other aromatic nuclei or by aliphatic chains of sufficient length to bring the molecular weight of the final base within the optimal zone.

With this aim in view 4-diphenylylpiperidinomethylcarbinol (III) was prepared by condensation of *p*-phenylphenacyl bromide with piperidine, followed by catalytic reduction

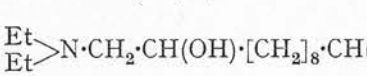
(III.) 



(IV.)

of the resulting ketone. The doubly substituted 4 : 4′-bis(β-*piperidino*-α-*hydroxyethyl*)-*diphenyl* (IV) was obtained similarly from 4 : 4′-*bis*-ω-*chloroacetyldiphenyl*. The inter-

mediate substance, diphenyl-4 : 4′-dicarboxylic acid, had a high melting point and was extremely sparingly soluble, so that to prepare its di-acid chloride it was necessary to use phosphorus pentachloride in molten diphenyl. The bis-ω-chloroacetyldiphenyl was then obtained from it through the bis-diazo-ketone. In this way also the corresponding *dichloro-ketones* were prepared from sebacic acid and from decanedicarboxylic acid. Both of these dichloro-ketones condensed readily with piperidine and with diethylamine, but the conversion of the resultant keto-amines into the corresponding carbinols could only be accomplished by prolonged reduction, a large excess of fresh platinum oxide being used. In this way 1 : 12-dipiperidino- and 1 : 12-bisdiethylamino-2 : 11-dihydroxydodecane (V) were obtained. Reduction by aluminium amalgam in neutral solution led to fission of the amino-ketone at the C–N linkage without reduction of the keto-group. The Ponndorf method also was impracticable. These results seem to suggest that α-keto-amines are more readily reduced to carbinols when the ketone group is conjugated with an aromatic system. The difficulty was avoided in the case of the tetradecane derivatives by the preparation of the ditertiary carbinol, 1 : 14-*dipiperidino*-2 : 13-*dihydroxy*-2 : 13-*dipropyltetradecane* (VI) by the action of propylmagnesium iodide.



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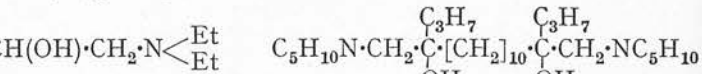
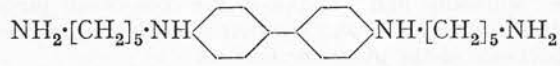


(VI.)

As no antiplasmodial activity was shown by any of these carbinols based on the type (I), attention was directed to the preparation of substances based on (II). Benzidine di-*p*-toluenesulphonate was successfully condensed in the presence of two molecular proportions of sodium hydroxide with γ-diethylaminopropyl chloride and with 1-chloro-5-benzamidopentane. On removal of the toluenesulphonate radicals, *NN*′-bis-(γ-diethylaminopropyl)benzidine and *NN*′-bis-(5″-aminoamyl)benzidine (VII) were obtained.



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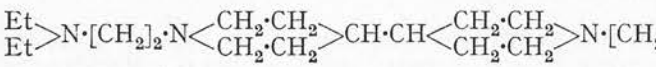

(VIII.)

Benzidine is a weak base of comparable strength with the aminoquinolines. As no antiplasmodial activity was found in these basic derivatives, attention was directed to the two very strong bases 4 : 4′-dipiperidyl and 2 : 4′-dipiperidyl, which are now available commercially. Each of these condensed readily with β-diethylaminoethyl chloride to give 1 : 1′-bis-(β-diethylaminoethyl)-4 : 4′-dipiperidyl (VIII) and 1 : 1′-bis-(β-diethylaminoethyl)-2 : 4′-dipiperidyl respectively. In a similar way tetrahydroquinoline was condensed with γ-diethylaminopropyl chloride to give 1-γ-diethylaminopropyltetrahydroquinoline. Finally, two aliphatic polyamines somewhat analogous to the naturally occurring base, spermine (Dudley, *Biochem. J.*, 1926, **20**, 1082), were prepared. 1 : 6-Diaminohexane and 1 : 10-diaminodecane were converted into their di-*p*-toluenesulphonyl derivatives, and these were alkylated with γ-diethylaminopropyl chloride. On removal of the toluenesulphonyl groups 1 : 6-bis-(γ-diethylaminopropylamino)hexane and 1 : 10-*bis*(γ-*diethylaminopropylamino*)*decane* (IX) were isolated. For comparison with these a base of somewhat simpler structure, 1 : 10-bis*iso*amylaminodecane (X) was prepared.

(IX.) $NEt_2 \cdot [CH_2]_3 \cdot NH \cdot [CH_2]_{10} \cdot NH \cdot [CH_2]_3 \cdot NEt_2$ $C_5H_{11} \cdot NH \cdot [CH_2]_{10} \cdot NH \cdot C_5H_{11}$ (X.)

As none of the basic substances of moderately high molecular weight described in this communication have exhibited antiplasmodial activity, it must be concluded that the quinoline nucleus of the cinchona alkaloids and of synthetic antimalarials is a potent factor in the production of antiplasmodial activity. The recent surprising results of Fulton (*Ann. Trop. Med. Parasit.*, 1940, **34**, 53) on the antiplasmodial activity of such a simple base as 1-diethylamino-4-aminopentane are contrary to this conclusion, but attempts to confirm his results have been unsuccessful.

Experimental.

4-Diphenylylpiperidinomethylcarbinol (III).—*p*-Phenylphenacyl bromide (4·0 g.) (Drake and Bronitsky, *J. Amer. Chem. Soc.*, 1930, **52**, 3716), dissolved in acetone, was added slowly with shaking to piperidine (2·5 g.) and after 1 hour the product was diluted with ether, and the precipitated piperidine hydrobromide collected. The filtrate was evaporated in a vacuum at room temperature, and the free base crystallised from ether; m. p. 86°. The *picrate*, crystallised from methanol–acetone, had m. p. 188° (Found: C, 59·5; H, 4·8; N, 11·4. $C_{19}H_{21}ON,C_6H_3O_7N_3$ requires C, 59·1; H, 4·7; N, 11·0%). The yield was almost quantitative. The free base was dissolved in ethanol, made just acid to Congo by addition of concentrated hydrochloric acid, and reduced, Adams's catalyst being used. Complete reduction was troublesome and the final product still contained some amino-ketone, which was removed most conveniently by recrystallisation of the free base from methanol. The pure hydroxyamine melted at 120° and gave a *methiodide*, m. p. 205° (Found: C, 56·7; H, 6·3. $C_{19}H_{23}ON,CH_3I$ requires C, 56·4; H, 6·1%). The hydrochloride melted at 243° (decomp.).

Diphenyl-4 : 4′-dicarboxylic Acid.—Benzidine (37 g.) was dissolved in concentrated hydrochloric acid (100 c.c.), and ice (400 g.) added. Sodium nitrite (about 90 c.c. of 30%) was added slowly until some free nitrous acid was present; the diazonium chloride solution was then neutralised by addition of saturated aqueous sodium carbonate. This mixture was added slowly with stirring to a solution of cuprous cyanide at 90° (from 171·6 g. of potassium cyanide and 159·8 g. of copper sulphate in 1060 c.c. of water). After cooling and standing overnight, the solid product was collected, dried, and extracted with alcohol (Soxhlet). The alcohol-soluble material (2·7 g.), m. p. 230—233°, was diphenyldinitrile and the alcohol-insoluble material (32·7 g.) appeared to be a copper complex, which was decomposed by warming on the water-bath for ½ hour with excess of concentrated hydrochloric acid. The acid was diluted, and the solid material filtered off, washed free from acid, and extracted with acetone (Soxhlet). The acetone-soluble extract crystallised readily, m. p. 230—233°, and was identical with the previous crop of diphenyldinitrile; total yield, 45%. The dinitrile (10·0 g.) was mixed with 70% sulphuric acid (90 c.c.) and refluxed for 45 minutes. The product when cold was poured into water, and the diphenyldicarboxylic acid filtered off, washed, and dried. The yield was 95%, and the product was sufficiently pure for conversion into the acid chloride.

Diphenyl-4 : 4′-dicarboxylic Acid Chloride.—Diphenyldicarboxylic acid (12·5 g.) was powdered, mixed with diphenyl (50 g.), heated under a reflux condenser to 150°, and powdered phosphorus pentachloride (21 g.) added slowly. After removal of the phosphorus oxychloride the diphenyl di-acid chloride, which was soluble in hot diphenyl, was purified by distilling off most of the diphenyl in a vacuum and pouring the residue into hot benzene. The acid chloride crystallised in long white needles sparingly soluble in cold benzene, m. p. 184° (yield, 80%). Purification was also attempted by distillation in a high vacuum, but resulted in considerable decomposition. Attempts to use thionyl chloride in place of phosphorus pentachloride were unsuccessful.

4 : 4′-Di-ω-chloroacetyldiphenyl.—Diphenyl di-acid chloride (8·0 g.) in solution in warm benzene (300 c.c.) was added slowly to a solution of diazomethane (from 20 g. of nitrosomethylurea) in ether. When the vigorous reaction had ceased, the precipitated diazo-ketone, m. p. 165° (decomp.), was collected and used immediately for the next stage without further purification. The diazo-ketone was suspended in benzene (250 c.c.), warmed to 40°, and a rapid stream of dry hydrogen chloride passed into the solution until no more nitrogen was evolved. The pale yellow, crystalline product was filtered off and recrystallised from dioxan; m. p. 226—227°, yield 5·2 g. (Found: C, 62·6; H, 3·9. $C_{16}H_{12}O_2Cl_2$ requires C, 62·5; H, 3·9%).

4 : 4′-Bis-(β-piperidino-α-hydroxyethyl)diphenyl (IV).—The dichloro-ketone (4·0 g.) from the previous preparation was finely powdered, suspended in chloroform (25 c.c.), heated to the b. p., and added all at once to a hot solution of piperidine (4·55 g.) in chloroform (25 c.c.). After heating in a water-bath at 50° for ½ hour, the chloroform was concentrated to 25 c.c., and ether (100 c.c.) added. The precipitated piperidine hydrochloride (2·8 g.) was removed, the filtrate evaporated in a vacuum, and the solid residue recrystallised from ethanol; m. p. 140° (Found: C, 77·0; H, 7·7. $C_{26}H_{32}O_2N_2$ requires C, 77·2; H, 7·9%). The *amino-ketone* was dissolved in ethanol, made just acid to Congo by addition of hydrochloric acid, and reduced, Adams's catalyst being used (630 c.c. of hydrogen). 4 : 4′-Bis-(β-piperidino-α-hydroxyethyl)-diphenyl dihydrochloride, sparingly soluble in hot absolute alcohol, was readily isolated from the solution after removal of the catalyst and from it was obtained the free *base* (3·7 g.), m. p. 158° after recrystallisation from methanol (Found: C, 76·4; H, 8·5. $C_{26}H_{36}O_2N_2$ requires C, 76·5; H, 8·8%).

1 : 12-*Dichloro*-2 : 11-*diketododecane*.—Sebacic acid (20 g.) was converted into the acid chloride by treatment with phosphorus pentachloride (2 mols.), and the acid chloride purified by distillation, b. p. 140—143°/2 mm. The yield was almost quantitative. The acid chloride was dissolved in dry ether and run slowly into an ethereal solution of excess of diazomethane (from 50 g. of nitrosomethylurea), and the mixture kept overnight. The ether and diazomethane were removed by gentle warming in a vacuum and the *diazo-ketone*, which crystallised, was collected, m. p. 91° after recrystallisation from benzene; it was sparingly soluble in ether. Yield, 19·3 g. (Found: N, 22·4. $C_{12}H_{18}O_2N_4$ requires N, 22·7%). The diazo-ketone was dissolved in the minimum quantity of benzene, and dry hydrogen chloride passed in rapidly until no more nitrogen was evolved. The white crystalline product, sparingly soluble in cold benzene, melted at 92° after recrystallisation; yield, 19·5 g. (Found: C, 53·9; H, 7·5; Cl, 27·0. $C_{12}H_{20}O_2Cl_2$ requires C, 53·9; H, 7·5; N, 26·6%).

1 : 12-*Dipiperidino*-2 : 11-*dihydroxydodecane*.—To a solution of piperidine (2·55 g.) in acetone (10 c.c.) was added, slowly, powdered dichlorodiketododecane (2·0 g.). The mixture was kept for 1 hour at 35°, then diluted with ether, and the precipitated piperidine hydrochloride (1·7 g.) collected. The filtrate was evaporated in a vacuum, and the low-melting solid residue dissolved immediately in ethanol, made just acid to Congo with hydrochloric acid, and reduced, Adams's platinum catalyst being used. Reduction was troublesome and could not be completed. The desired amino-alcohol was isolated by fractional crystallisation from light petroleum, in which it was much less soluble than the amino-ketone. The amino-alcohol melted at 78° and gave a *dipicrate*, m. p. 152° (Found: C, 49·6; H, 5·8; N, 13·4. $C_{22}H_{44}O_2N_2,2C_6H_3O_7N_3$ requires C, 49·4; H, 6·0; N, 13·5%). The recovered unreduced ketone melted at 43°.

1 : 12-*Diethylamino*-2 : 11-*dihydroxydodecane*.—The method of condensation of the chloroketone (2·0 g.) and diethylamine was as in the previous experiment. Reduction with Adams's catalyst was again troublesome. In order to separate the ketone from the alcohol the product of the reduction was treated with Girard's " reagent P " and separated into water-soluble and ether-soluble fractions. From the ether-soluble fraction an oil was obtained (1·1 g.) giving a crystalline *dipicrate*, m. p. 121°, from alcohol (Found: N, 14·0. $C_{20}H_{44}O_2N_2,2C_6H_3O_7N_3$ requires N, 13·9%).

1 : 14-*Dichloro*-2 : 13-*diketotetradecane*.—The method was the same as that used in the preparation of dichlorodiketododecane. *n*-Decanedicarboxylic acid was converted into the acid chloride and treated with diazomethane. The *diazo-ketone* after crystallisation from benzene melted at 96° (Found: C, 60·5; H, 7·9. $C_{14}H_{22}O_2N_4$ requires C, 60·4; H, 7·9%). The diazo-ketone, treated with hydrochloric acid, gave the *chloro-ketone*, m. p. 97° (Found: C, 57·2; H, 8·4. $C_{14}H_{24}O_2Cl_2$ requires C, 56·9; H, 8·2%). The yield was 17·0 g. from 20·0 g. of decanedicarboxylic acid.

1 : 14-*Dipiperidino*-2 : 13-*diketotetradecane*.—The chloro-ketone (2·9 g.), condensed with piperidine (3·2 g.) in the same way as in the case of the lower homologue, gave crystalline dipiperidinodiketotetradecane (2·9 g.), but reduction of this compound could not be carried out satisfactorily either with Adams's catalyst or with palladised charcoal. An attempt was made to carry out the reduction with neutral aluminium amalgam. The base (m. p. 48°) was exactly neutralised with hydrochloric acid and kept for 3 hours in aqueous solution over a large excess of aluminium amalgam which had been prepared by treating clean aluminium turnings with a saturated ethereal solution of mercuric chloride for a few seconds and then washing thoroughly with ether (cf. Weitz, König, and Wistinghausen, *Ber.*, 1924, **57**, 167). The residual aluminium amalgam was removed and washed with alcohol and ether, and the filtrate and washings acidified slightly and extracted with ether. The extract gave a white solid, which was crystallised from light petroleum; m. p. 75° (yield, 150 mg.) (Found: C, 73·9; H, 11·5. $C_{14}H_{26}O_2$ requires C, 74·3; H, 11·5%). The compound gave a precipitate with 2 : 4-dinitrophenylhydrazine and was apparently 2 : 13-*diketotetradecane*. The aqueous fraction was made alkaline and extracted with ether. From the ether a base (160 mg.) was obtained from which no crystalline derivative could be prepared.

1 : 14-*Dipiperidino*-2 : 13-*dihydroxy*-2 : 13-*dipropyltetradecane* (VI).—Dipiperidinodiketotetradecane (2·7 g.) in ether (25 c.c.), prepared as described above, was added slowly to a Grignard reagent, prepared from magnesium (0·33 g.) and propyl bromide (1·7 g.) in ether (25 c.c.), with vigorous stirring. After ½ hour, cold 3N-hydrochloric acid was added and the resulting solution, after being washed with ether, was made alkaline with ammonia and extracted with ether. The extract gave 2·6 g. of a colourless oil. This product could not be purified as a crystalline derivative and was therefore distilled, b. p. 230—240°/0·3 mm. The substance

was not dehydrated by this process and analysis showed that it was mainly the desired *dicarbinol* (Found : C, 74·5; H, 12·4; N, 6·4. $C_{30}H_{60}O_2N_2$ requires C, 75·3; H, 12·1; N, 5·9%). No crystalline derivative was obtained.

Benzidine Di-p-*toluenesulphonate.*—The preparation was carried out as described by Willstätter and Kalb (*Ber.*, 1904, **37**, 3772), but the method of purification of the crude amide was modified. The crude product from the Schotten–Baumann reaction was dried and extracted continuously with alcohol (Soxhlet) until the m. p. of the material dissolving in the alcohol was about 140°. The solid remaining in the thimble was crystallised from acetone (charcoal); m. p. 241—243°, yield 70%.

NN'-Bis-(γ-diethylaminopropyl)benzidine.—Benzidine ditoluenesulphonate (6·56 g.), γ-diethylaminopropyl chloride (4·0 g.), sodium hydroxide (1·06 g.), and 75% alcohol (200 c.c.) were heated in a sealed bottle for 24 hours in a boiling water-bath. The solvent was removed from the product, which was then shaken with water, and the water decanted and evaporated (1·34 g. of sodium chloride). The water-insoluble gum was heated in a sealed tube for 4 hours at 180° with glacial acetic acid (10 c.c.) and concentrated hydrochloric acid (25 c.c.). The product was evaporated to dryness, dissolved in water, filtered from insoluble residue, made alkaline, and extracted repeatedly with benzene. The benzene-soluble oil distilled at 230—250°/0·9 mm. The distillate (2·2 g.) gave a crystalline *tetrahydrobromide*, m. p. 260° (decomp.), from methanol (Found : C, 42·3; H, 6·4; N, 7·8. $C_{26}H_{40}N_4,4HBr$ requires C, 42·6; H, 6·1; N, 7·7%).

NN'-Bis-(5''-aminoamyl)benzidine (VII).—Benzidine ditoluenesulphonate (6·56 g.), sodium hydroxide (1·06 g.), 1-chloro-5-benzamidopentane (Ainley and King, *Proc. Roy. Soc.*, 1938, **125**, 68), and 50% aqueous acetone (50 c.c.) were mixed thoroughly and heated in a sealed tube at 150—160° for 3 hours. When cold the aqueous acetone was decanted from the heavy oily product, which was washed with water. NN'-*Bis-(5''-benzamidoamyl)benzidine di*-p-*toluenesulphonate* crystallised on trituration with acetone and on recrystallisation from the same solvent had m. p. 192°; yield, 6·5 g. (Found : C, 68·8; H, 6·0. $C_{50}H_{54}O_6N_4S_2$ requires C, 69·1; H, 6·0%). This product (4·25 g.) was suspended in glacial acetic acid (25 c.c.), and hydrogen chloride passed into the mixture until the solid dissolved. The solution was heated with concentrated hydrochloric acid (10 c.c.) in a Carius tube at 180—190° for 3½ hours. The liquid was evaporated to dryness, the residue dissolved in water, and benzoic acid (0·86 g.) extracted with ether. The aqueous layer was filtered and evaporated to dryness. The resultant yellow gum crystallised when triturated with absolute alcohol, and was recrystallised from methanol; m. p. 270° (decomp.), yield 1·8 g. The *tetrahydrochloride* was hygroscopic (Found : C, 52·4; H, 6·8; N, 11·3. $C_{22}H_{31}N_4,4HCl$ requires C, 52·8; H, 7·4; N, 11·2%).

1 : 1'-*Bis-(β-diethylaminoethyl)*4 : 4'-*dipiperidyl* (VIII).—Dipiperidyl (4·2 g.), β-diethylaminoethyl chloride (6·8 g.), and alcohol (25 c.c.) were heated in a sealed tube at 100° for 18 hours. The alcohol was removed, and the product dissolved in water, made alkaline, and extracted with chloroform. The oil remaining on evaporation of the extract was distilled at 0·3 mm. and the portion boiling between 200° and 230° (3·04 g.) was further fractionated by the method of differing basicities (King, J., 1919, **117**, 991). The three main portions (2·12 g.) were identical and gave a *tetrapicrate* very sparingly soluble in acetone, m. p. 250° (decomp.) (Found : C, 43·5; H, 4·7. $C_{22}H_{46}N_2,4C_6H_3O_7N_3$ requires C, 43·1; H, 4·5%).

1 : 1'-*Bis-(β-diethylaminoethyl)*-2 : 4'-*dipiperidyl.*—A mixture of 2 : 4'-dipiperidyl (4·2 g.), β-diethylaminoethyl chloride, and alcohol (25 c.c.) was treated as described above (14 hours' heating). The oil obtained from the chloroform extract gave on distillation two fractions, b. p. 150—160°/0·5 mm., and 200—210°/0·5 mm. The latter, b. p. 205—210°/0·5 mm. on redistillation, gave a crystalline *tetrapicrate* (3·5 g.) moderately easily soluble in acetone, m. p. 170° (Found : C, 43·2; H, 4·5; N, 17·3. $C_{22}H_{46}N_4,4C_6H_3O_7N_3$ requires C, 43·1; H, 4·5; N, 17·6%).

1-γ-*Diethylaminopropyltetrahydroquinoline.*—Tetrahydroquinoline (8·0 g.) and γ-diethylaminopropyl chloride (4·0 g.) were heated together at 100° without solvent for 10 hours, aqueous alkali added, and the base extracted with ether. The oil obtained gave a main fraction, b. p. 190—192°/11·0 mm., 192°/10·0 mm. on redistillation (yield, 6·2 g.), which gave a crystalline *dipicrate* from ethanol, m. p. 147° (Found : C, 47·9; H, 4·5; N, 16·4. $C_{16}H_{23}N_2,2C_6H_3O_7N_3$ requires C, 47·9; H, 4·2; N, 16·0%).

1 : 6-*Bis*-p-*toluenesulphonylaminohexane.*—1 : 6-Diaminohexane (4·0 g.) was added to *p*-toluenesulphonyl chloride (13·5 g.) previously covered with a little water, and aqueous sodium hydroxide (3·0 g. in 15 c.c.) added. The mixture was heated on the water-bath for 1 hour and then cooled rapidly and shaken vigorously. The white solid was recrystallised from alcohol; m. p. 152°, yield 13·9 g. (Found : C, 56·7; H, 6·7. $C_{20}H_{28}O_4N_2S_2$ requires C, 56·6; H, 6·7%).

1 : 6 - *Bis* - (*γ* - *diethylaminopropylamino*)*hexane*.—1 : 6 - Bis-*p*-toluenesulphonylaminohexane (13·0 g.) was dissolved in alcohol (200 c.c. of 70%) containing sodium hydroxide (2·45 g.; 2 mols.), and to this was added *γ*-diethylaminopropyl chloride (9·17 g.; 2 mols.). The mixture was heated in a sealed bottle at 100° for 16 hours, then evaporated to dryness, the residue dissolved in absolute alcohol, and the precipitated sodium chloride (2·55 g.) filtered off. On evaporation of the alcohol a colourless syrup was obtained, which was heated with glacial acetic acid (20 c.c.) and concentrated hydrochloride acid (4·5 c.c.) in a sealed tube at 180° for 2 hours. After cooling, the toluene formed was removed, and the acid solution evaporated. The brown gum was dissolved in water (20 c.c.), made alkaline with 50% potassium hydroxide solution, and shaken with ether. Three layers formed, the bottom one being aqueous alkali. This was run off, and water added to the two remaining layers in sufficient quantity to dissolve the oily layer; the aqueous and the ethereal layer were then worked up separately. The latter gave an uncrystallisable oil (3·5 g.). The aqueous solution was extracted continuously with chloroform for 12 hours, and the chloroform dried and evaporated, giving 4·7 g. of a base, which on fractional distillation at 0·5 mm. gave 0·4 g. up to 135°, 2·7 g. at 135—140°, and 1·0 g. at 160—220°. The middle fraction gave a hygroscopic crystalline *hydrobromide*, m. p. 64°, which was purified with difficulty by crystallisation from alcohol–acetone (Found: C, 34·1; H, 7·8; N, 8·6. $C_{20}H_{46}N_4,4HBr$ requires C, 35·8; H, 7·6; N, 8·4%).

1 : 10-*Bis*-p-*toluenesulphonylaminodecane*.—Sebaconitrile was reduced by sodium and alcohol to 1 : 10-diaminodecane in 85% yield (Phookan and Krafft, *Ber.*, 1892, **25**, 2253). The diaminodecane (11·2 g.) was treated with *p*-toluenesulphonyl chloride (24·1 g.) and sodium hydroxide (5·1 g.) as described for the preparation of the lower homologue, and the *product* crystallised from alcohol; m. p. 129°, yield 28·2 g. (Found: C, 60·3; H, 7·6. $C_{24}H_{36}O_4N_2S_2$ requires C, 60·0; H, 7·5%).

1 : 10-*Bis*-(*γ*-*diethylaminopropylamino*)*decane* (IX).—1 : 10-Bis-*p*-toluenesulphonylaminodecane (6·24 g.), *γ*-diethylaminopropyl chloride (3·9 g.), sodium hydroxide (1·05 g.), and 70% alcohol (100 c.c.) were heated in a sealed bottle for 16 hours. The product was evaporated to dryness, the residue dissolved in absolute alcohol, the sodium chloride (1·4 g.) removed, and the filtrate evaporated to a clear gum. This was heated with glacial acetic acid (10 c.c.) and concentrated hydrochloric acid (25 c.c.) in a sealed tube at 180—190° for 4 hours. The product was evaporated to dryness, and the residue dissolved in water, made strongly alkaline with sodium hydroxide, cooled in ice, and extracted rapidly several times with ethyl acetate. The ethyl acetate was evaporated, and the residue dissolved in benzene and filtered while hot. The filtrate was evaporated to a brown oil, which was distilled at 1·5 mm. and separated into two fractions, b. p. up to 150° and b. p. 178—184°. The former (0·67 g.) was recovered diaminodecane; the higher-boiling fraction, after redistillation, gave a very hygroscopic hydrobromide, m. p. 142—143° (crude), which could not be readily purified. The free *base* was preferred for analysis (Found: C, 73·0; H, 13·7; N, 13·7. $C_{24}H_{54}N_4$ requires C, 72·7; H, 13·7; N, 14·0%).

1 : 10-*Bis*isoa*mylaminodecane* (X).—1 : 10-Bis-*p*-toluenesulphonylaminodecane (6·24 g.), *iso*amyl bromide (4·0 g.), sodium hydroxide (1·05 g.), and 70% alcohol (100 c.c.) were heated together as in the previous experiment, and sodium bromide (2·2 g.) removed from the product. Hydrolysis of the alcohol-soluble gum was carried out as in the previous experiment and the product, which was tarry, was poured into hot water; the liquid was boiled for several minutes and filtered hot. The white crystalline precipitate which formed on cooling was 1 : 10-*bis*-iso*amylaminodecane dihydrochloride* (1·5 g.), m. p. 318° (Found: C, 62·3; H, 11·1. $C_{20}H_{44}N_2,2HCl$ requires C, 62·3; H, 12·0%). From the aqueous mother-liquor, 1·1 g. of diaminodecane hydrochloride were recovered.

γ-Diethylaminopropyl Chloride.—The method of Magidson (*Arch. Pharm.*, 1933, **271**, 569) was successfully employed, but the yields in the first stages were not as good as reported, being only about 50% in the condensation of diethylamine and trimethylene chloroacetate with hydrolysis to diethylaminopropanol. The b. p. of *γ*-diethylaminopropyl chloride was found to be 75—76°/29 mm. and not 85°/28 mm. as recorded by Magidson.

I desire to express my thanks to Miss Ann Bishop, D.Sc., working in the laboratory of Prof. D. Keilin, F.R.S., at the Molteno Institute, Cambridge, for carrying out the biological tests. I am also indebted to Mr. N. Schunmann for technical assistance.

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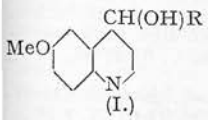
72. *Antiplasmodial Action and Chemical Constitution. Part V. Carbinolamines derived from 6-Methoxyquinoline.*

By Harold King and Thomas S. Work.

The aim of this investigation was the search for antiplasmodial substances based on the formula of quinine but of simpler structure. The preparation of a number of unsymmetrical dialkylamines is described and, from these, 1 : 2-carbinolamines have been synthesised containing the 6-methoxyquinoline nucleus. Although the structure of these carbinolamines was very close to that of active antiplasmodials described in Part III, these bases proved to be inactive when tested on bird-malaria in canaries.

In Part III (King and Work, J., 1940, 1307), it was shown that there was antiplasmodial activity, as tested on bird-malaria in canaries, in the *N*-dibutyl-, -diamyl- and -dihexyl-aminomethyl-6-methoxy-4-quinolylcarbinols (I, R = $CH_2 \cdot NR_1R_2$). The lower and the higher dialkyl homologues were, however, inactive. There was thus a zone of activity where the sum of the carbon atoms of the *N*-alkyl groups lay between eight and twelve. The present communication describes the synthesis of seven members of a series of secondary amines, in which the groups R_1 and R_2 are different from each other, and their combined carbon atom content is not greater than 12 and not less than 8. It has only been found possible to prepare bases of the structure (I, R = $CH_2 \cdot NR_1R_2$) from four of these unsymmetrical secondary bases. In the other cases catalytic reduction of the intermediate quinolylketo-bases was followed by fission of the basic group with formation of methyl-6-methoxy-4-quinolylcarbinol (I, R = CH_3).



(I.)

Ethyl-, *propyl-*, *butyl-* and *amyl-hexylamines* were prepared by the action of the appropriate alkyl iodides

or bromides on benzylhexylamine. The benzyl group was then removed by catalytic reduction in acetic acid at 70° in the presence of platinum oxide:

$$C_6H_{13}\cdot NH\cdot CH_2Ph + R\cdot Hal \longrightarrow C_6H_{13}\cdot NR\cdot CH_2Ph \longrightarrow C_6H_{13}\cdot NHR + Ph\cdot CH_3$$

In a similar manner *ethyl-* and *propyl-nonylamines* were obtained. Difficulties were, however, encountered in the preparation of *methylnonylamine*. When methyl iodide and *benzylnonylamine* were allowed to react, the products were mainly unchanged base and *benzyldimethylnonylammonium iodide*. This quaternary salt was, however, converted into the hydroxide and then into the hydrosulphide, following Clarke's method (J., 1913, **103**, 1689). On evaporation of the solution of this hydrosulphide under reduced pressure the products were *dimethylnonylamine*, benzylmercaptan and dibenzyl sulphide, the benzyl group showing a preference to be eliminated over the methyl group. This might have been anticipated from the known tendency for benzyl

$$\left[\begin{matrix}Me\\Me\end{matrix}\!\!>\!N\!<\!\begin{matrix}C_9H_{19}\\CH_2Ph\end{matrix}\right]SH \longrightarrow Me_2N\cdot C_9H_{19} + Ph\cdot CH_2\cdot SH + Ph\cdot CH_2\cdot S\cdot S\cdot CH_2Ph$$

groups to be eliminated over other groups in many reactions (compare Snyder and Speck, *J. Amer. Chem. Soc.*, 1939, **61**, 669, 2895, where several references are given). This easy elimination of benzyl groups limits the applicability of Clarke's useful method for the demethylation of quaternary ammonium salts to cases where the benzyl group is absent.

When nonyl iodide and methylamine were allowed to interact in methyl-alcoholic solution at 100°, methylnonylamine was formed, but the main product was *methyldinonylamine*. Methylnonylamine was eventually obtained in good yield by an application of Decker's method. Benzaldehyde and nonylamine were condensed to give *benzylidenenonylamine*, which on methylation gave an oily methiodide. On digestion of the latter with 90% alcohol and treatment with mineral acid benzaldehyde was liberated to ether, and the methylnonylamine recovered from the acid liquor.

The results of the tests on bird malaria due to *Plasmodium relictum* in canaries, of the carbinolamines described in this communication are shown below.

Substance.	Dose in mg. per 20 g. of body weight.	Day of appearance of parasites in blood.	Remarks.
Ethylhexylaminomethyl-6-methoxy-4-quinolylcarbinol	6 × 5 *	5	M.T.D.†
	1 × 5 + 5 × 2·5	6	
Propylhexylaminomethyl-6-methoxy-4-quinolylcarbinol	6 × 5	5	M.T.D.
	6 × 2·5	6	
Butylhexylaminomethyl-6-methoxy-4-quinolylcarbinol	6 × 5	5	M.T.D.
	6 × 5	5	
Methylnonylaminomethyl-6-methoxy-4-quinolylcarbinol	1 × 2·5 + 5 × 1·25	6	M.T.D.
	1 × 2·5 + 5 × 1·25	6	
2′ : 2′ : 6′-Trimethylpiperidinomethyl-6-methoxy-4-quinolylcarbinol	6 × 5	5	M.T.D.
	6 × 5	5	
Quinine dihydrochloride	6 × 2·5	10	
Control birds		5, 6, 5	

* This means a dose of 5 mg. was given daily for 6 days, the first dose being administered 4 hours after inoculation with malaria.

† M.T.D. = maximum tolerated dose.

In Part III (p. 139) it was found that piperidinomethyl-6-methoxy-4-quinolylcarbinol was inactive on bird malaria. It was thought possible that activity might be restored by bringing up the carbon content to the level shown necessary in the symmetrical secondary bases, that is, between eight and twelve carbon atoms. Accordingly 2′ : 2′ : 6′-trimethylpiperidinomethyl-6-methoxy-4-quinolylcarbinol (I, R = $\cdot N\!<\!\begin{matrix}CMe_2\text{—}CH_2\\CHMe\cdot CH_2\end{matrix}\!>\!CH_2$) was synthesised, but it proved to be without action in the dose shown in the table.

Experimental.

*Benzyl-*n*-hexylamine.*—This base is a by-product in the preparation of benzyldi-*n*-hexylamine (King and Work, J., 1940, 1313). It may be obtained as the main product by the following process: Hexyl bromide (66·0 g.; 1 mol.) and benzylamine (85·6 g.; 2 mols.) after about an hour deposited benzylamine hydrobromide. The mixture was heated on the boiling water-bath for an hour, cooled, and diluted with dry ether, and the benzylamine hydrobromide collected. The ethereal solution was shaken with aqueous sodium hydroxide, dried over potassium hydroxide, then fractionated, giving benzylamine (19·0 g.), b. p. 80—100°/18 mm., benzyl-*n*-hexylamine (51·6 g.), b. p. 142—160°/18 mm. (47 g. on redistillation, b. p. 149—150°/18 mm.; yield, 62% on the hexyl bromide used), and benzyldi-*n*-hexylamine 6·7 g.), b. p. 160—180°/18 mm.

*Benzyl-*n*-butylhexylamine.*—Benzylhexylamine (19·1 g.), *n*-butyl bromide (15·1 g.), and potassium hydroxide (7·7 g.) were heated at 140° for 5 hours. The product was cooled, diluted with aqueous alkali, and extracted with ether. The main non-ethereal fraction (22·75 g.), b. p. 165—171°/18 mm., on refractionation through a column gave *benzyl-*n*-butylhexylamine* (17·4 g.), b. p. 170°/18 mm. (Found: C, 82·7; H, 11·9; N, 5·7. $C_{17}H_{29}N$ requires C, 82·6; H, 11·7; N, 5·7%).

n-*Butylhexylamine.*—Benzylbutylhexylamine (24·7 g.), reduced at 70° (see amyl analogue, below), gave *butylhexylamine* (13·6 g.), b. p. 201°/738 mm. (Found: C, 76·1; H, 14·8; N, 9·0. $C_{10}H_{23}N$ requires C, 76·4; H, 14·6; N, 8·9%). The *hydrochloride* crystallised from acetone in filmy leaflets, m. p. 268° (Found: C, 62·0; H, 12·6. $C_{10}H_{23}N,HCl$ requires C, 62·0; H, 12·5%).

Benzyl-n-amylhexylamine.—Benzylhexylamine (19·1 g.), *n*-amyl bromide (16·6 g.), and potassium hydroxide (17·7 g.) were refluxed at 140° for 3 hours. The mixture obtained in ethereal solution after addition of aqueous caustic alkali gave (1) b. p. up to 100°/15 mm. (hexyl bromide), (2) 5·3 g., b. p. 150—170°/15 mm., (3) 18·0 g., b. p. 170—180°/15 mm. On refractionation (2) and (3) gave *benzyl-n-amylhexylamine* (16·8 g.), b. p. 175—177°/15 mm. (79% yield) (Found: C, 82·3; H, 11·9; N, 5·5. $C_{18}H_{31}N$ requires C, 82·7; H, 12·0; N, 5·4%).

n-*Amylhexylamine.*—Benzylamylhexylamine (26·1 g.) in glacial acetic acid (30 c.c.) and platinum oxide (0·4 g.) was hydrogenated at 70° for 12 hours, and the filtered solution diluted with water and made strongly alkaline. The dried ethereal extract gave n-*amylhexylamine* (14·4 g.), b. p. 108°/15 mm., 216—218°/763 mm. (Found: N, 7·9. $C_{11}H_{25}N$ requires N, 8·2%). The *hydrochloride* separated from acetone in pearly leaflets, m. p. 275—276° (Found: C, 63·8; H 12·4. $C_{11}H_{25}N$,HCl requires C, 63·6; H, 12·6%).

Benzyl-n-propylhexylamine, prepared from benzylhexylamine (19·1 g.), *n*-propyl iodide (17·0 g.), and potassium hydroxide (7·7 g.) at 140° (external bath), had b. p. 149—155°/15 mm. (yield, 20·5 g.) and on refractionation 155°/15 mm. (Found: C, 82·3; H, 11·3; N, 6·2. $C_{16}H_{27}N$ requires C, 82·3; H, 11·7; N, 6·0%).

n-*Propylhexylamine* obtained from benzylpropylhexylamine (34·9 g.) by reduction at 70°, had b. p. 171—181°/753 mm.; yield, 15·4 g. (Found: C, 75·2; H, 15·0; N, 9·5. $C_9H_{21}N$ requires C, 75·5; H, 14·7; N, 9·8%). The very soluble *hydrochloride*, obtained from hydrogen chloride and a low-boiling petroleum solution of the base, crystallised from acetone in pearly leaflets, m. p. 243° (Found: C, 60·2; H, 12·1. $C_9H_{21}N$,HCl requires C, 60·1; H, 12·3%).

Benzylethylhexylamine.—Benzylhexylamine (9·55 g.), ethyl iodide (8·6 g.), and potassium hydroxide (3·85 g.) were heated in sealed tubes at 140—150° for 4 hours. An ethereal extract of the basified product gave *benzylethylhexylamine* (15·6 g.), b. p. 145°/13 mm. (Found: C, 81·9; H, 11·6; N, 6·6. $C_{15}H_{25}N$ requires C, 82·3; H, 11·4; N, 6·4%).

Ethylhexylamine (10·9 g.), obtained from the above base (29 g.) by reduction at 70° and isolated by repeated extraction with ether of the strongly basified mixture, had b. p. 158°/743 mm. (Found: C, 74·2; H, 14·8; N, 10·6. $C_8H_{19}N$ requires C, 74·2; H, 14·8; N, 10·6%). The *hydrochloride* (see propyl analogue) crystallised in pearly leaflets, m. p. 191° (Found: C, 58·2; H, 11·9. $C_8H_{19}N$,HCl requires C, 58·0; H, 12·2%).

Benzyl-n-nonylamine (30·1 g.), obtained from benzylamine (42·8 g.) and nonyl bromide (41·4 g.) (see hexyl analogue), had b. p. 179°/12 mm. (Found: C, 82·0; H, 11·6; N, 6·2. $C_{16}H_{27}N$ requires C, 82·3; H, 11·7; N, 6·0%). *Benzyldinonylamine* (7·0 g.), b. p. 240°/12 mm., was also obtained (Found: C, 83·4; H, 12·3; N, 4·2. $C_{25}H_{45}N$ requires C, 83·5; H, 12·6; N, 3·9%). *Benzylnonylamine hydrochloride* separated from water in needles, m. p. 199—200° (Found: C, 70·9; H, 10·3. $C_{16}H_{27}N$,HCl requires C, 71·2; H, 10·5%).

Benzyl-n-propylnonylamine (32·5 g.), obtained from benzylnonylamine (34·9 g.), *n*-propyl iodide (25·5 g.), and potassium hydroxide (11·5 g.) at 130°, had b. p. 185°/13 mm. (Found: C, 83·4; H, 12·0; N, 5·4. $C_{13}H_{33}N$ requires C, 82·8; H, 12·1; N, 5·1%).

Propylnonylamine (12·3 g.), obtained by reduction of benzylpropylnonylamine (32·5 g.), had b. p. 119°/14 mm. (Found: C, 77·7; H, 14·5. $C_{12}H_{27}N$ requires C, 77·8; H, 14·7%). The *hydrochloride* crystallised from dilute hydrochloric acid in pearly scales, m. p. 237° (Found: C, 64·3; H, 13·0. $C_{12}H_{27}N$,HCl requires C, 64·9; H, 12·7%).

Benzylethylnonylamine.—A mixture of benzylnonylamine (23·3 g.; 1 mol.) and ethyl iodide (17·16 g.; 1 mol.) became warm after 30 minutes and crystals separated. Potassium hydroxide (7·7 g.) was added, the mixture heated on the water-bath for 4 hours, and the product treated with water and ether. The solid (2·2 g.) that separated crystallised from ethyl acetate in white plates, m. p. 64—65°, of *benzyldiethylnonylammonium iodide* (Found: C, 57·2; H, 8·9; N, 3·4. $C_{20}H_{36}NI$ requires C, 57·5; H, 8·7%). The ethereal extract on fractional distillation gave *benzylethylnonylamine* (22·1 g.), b. p. 178°/11 mm. (Found: C, 82·7; H, 11·8. $C_{18}H_{31}N$ requires C, 82·7; H, 12·0%).

Ethylnonylamine (yield, 84·3%), obtained by catalytic reduction of benzylethylnonylamine (22·1 g.) at 70°, had b. p. 103°/14 mm. (Found: C, 77·1; H, 14·5. $C_{11}H_{25}N$ requires C, 77·1; H, 14·7%). The *hydrochloride* crystallised from acetone in pearly leaflets, m. p. 200—201° (Found: C, 63·8; H, 12·6. $C_{11}H_{25}N$,HCl requires C, 63·6; H, 12·6%).

Reaction between Benzylnonylamine and Methyl Iodide.—Benzylnonylamine (16 g.) and methyl iodide (10·7 g.; 1·1 mols.), gradually mixed, reacted vigorously, giving mainly unchanged benzylnonylamine (9·25 g.) and *benzyldimethylnonylammonium iodide*. The latter crystallised from ethyl acetate in plates (4·9 g.), m. p. 89° (Found: C, 54·9; H, 8·3; N, 3·6. $C_{18}H_{32}NI$ requires C, 55·5; H, 8·3; N, 3·6%).

A solution of the methochloride [obtained from the quaternary iodide (14·1 g.)] in water (100 c.c.) was treated with silver oxide, filtered, saturated with hydrogen sulphide, and evaporated to a syrup under reduced pressure at 50°; the residue was re-evaporated three times with a little absolute ethyl alcohol and then heated in a boiling water-bath for 4 hours. The product was now ether-soluble and on fractional distillation gave (*a*) 5·2 g., b. p. 97—99°/14 mm., and (*b*) 1·7 g., b. p. 170—180°/14 mm. As fraction (*a*) gave a nitroprusside reaction for thiol groups, it was dissolved in ether and extracted with aqueous sodium hydroxide. The ethereal solution now gave a sulphur-free product, b. p. 209°/741 mm., which proved to be *dimethylnonylamine* (Found: C, 77·1; H, 14·7. $C_{11}H_{25}N$ requires C, 77·7; H, 14·1%). The *methiodide* separated from water in filmy leaflets, m. p. 170° (Found: C, 45·9; H, 9·1. $C_{12}H_{28}NI$ requires C, 46·0; H, 9·0%). The thiol isolated from the sodium hydroxide extract by acidification in the presence of ether was benzylthiol (0·4 g.), b. p. 195—197°; the 2 : 4-dinitrophenyl thioether prepared from it had m. p. 128°; Bost, Turner, and Norton (*J. Amer. Chem. Soc.*, 1932, **54**, 1985) give m. p. 130° (Found: C, 54·2; H, 3·9. Calc.: C, 53·8; H, 3·7%).

Fraction (*b*) solidified and crystallised from methyl alcohol in bold plates, m. p. and mixed m. p. with dibenzyl sulphide 47°. The dibenzylsulphine-*p*-toluenesulphonylimine prepared from it had m. p. 191° (Mann and Pope, J., 1922, **121**, 1053, give m. p. 193°).

Methylnonylamine.—(*a*) Nonyl bromide (20·7 g.) was converted into nonyl iodide (24·0 g.), b. p. 116°/15 mm. by Finkelstein's method. The iodide was mixed with methylamine (20 c.c.; 33% solution) in methyl alcohol and heated at 100° for 6 hours. The product was treated with aqueous sodium hydroxide, and the excess of methylamine and alcohol distilled off on the water-bath. The product extracted from the alkaline solution by ether was fractionally distilled, giving methylnonylamine (2·6 g.), b. p. 92—94°/15 mm., identified as its hydrochloride, m. p. 179—180° (see below), and *methyldinonylamine* (10·0 g.), b. p. 190—192°/15 mm. (Found: C, 80·5; H, 14·4. $C_{19}H_{41}N$ requires C, 80·6; H, 14·5%).

(*b*) *By Decker's method.* Nonylamine (9·0 g.), b. p. 85°/13 mm., was condensed with benzaldehyde (7 g.; 1·1 mols.) by heating on the water-bath for 1 hour. The *benzylidenenonylamine* was distilled under reduced pressure (yield, 13·6 g.), b. p. 179°/14 mm. (Found: C, 82·5; H, 11·2; N, 6·6. $C_{16}H_{25}N$ requires C, 83·1; H, 10·8; N, 6·3%). This was mixed with methyl iodide (8·4 g.; 1·1 mols.) and after 1 hour heated on the water-bath for 3 hours. The dark red, viscous oil was diluted with 90% ethyl alcohol (40 c.c.) and digested on the water-bath for 1 hour; the alcohol was then removed by distillation, the residue acidified, and the benzaldehyde removed by ether extraction. *Methylnonylamine* (6·6 g.), recovered from the acid solution, had b. p. 95°/14 mm. (Found: N, 9·1. $C_{10}H_{23}N$ requires N, 9·1%). The *hydrochloride* separated from acetone in needles or plates, m. p. 180—181° (Found: C, 61·6; H, 12·6. $C_{10}H_{23}N$,HCl requires C, 62·0; H, 12·5%).

Quininic Acid.—Thielepape's procedure (*Ber.*, 1939, **72**, 1432) was in the main followed, but it was found advan-

tageous when preparing α-ethoxalyl-*N*-methyl-*p*-methoxyacetanilide to dissolve the *N*-methyl-*p*-methoxyacetanilide (17·9 g.) in the ethyl oxalate (13·4 c.c.) and to add this slowly to a hot solution of sodium ethoxide (2·3 g. of sodium in 11·6 c.c. of absolute ethyl alcohol). Heating was continued for 2 hours, and the product (16·5 g.) isolated and purified as described by Thielepape.

Conversion of ethyl 2-chloroquininate into quininic acid as described by Thielepape was laborious and the yields were not satisfactory. The following process gave better results: Ethyl 2-chloroquininate (53 g.) was boiled vigorously with sodium hydroxide (9·0 g. in 1 l. of water) for 2 hours, the solution concentrated under reduced pressure to 350 c.c., sodium hydroxide (9·0 g.) and a palladium–strontium carbonate catalyst (2·0 g.) added, and the solution hydrogenated at 5 atms. for 45 minutes. The catalyst was collected and re-used for subsequent batches. Quininic acid was precipitated by addition of hydrochloric acid until the reaction of the solution was definitely acid to Congo-paper. The yield was almost quantitative.

Ethylhexylaminomethyl-6-methoxy-4-quinolylcarbinol.—Powdered 6-methoxy-4-quinolyl bromomethyl ketone hydrobromide (6·6 g.) (King and Work, *loc. cit.*) was added slowly to ethylhexylamine (7·1 g.) in acetone (10 c.c.). After 1 hour the mixture was warmed at 45° for 45 mins. and diluted with dry ether, and the ethylhexylamine hydrobromide collected (7·0 g.). The ethereal solution was washed with water, and the ether removed in a vacuum. A portion of the residual oil (2·5 g.) was dissolved in ethyl alcohol, concentrated hydrochloric acid added until the solution was acid to Congo-paper, and the product reduced, platinum oxide being used as catalyst (hydrogen consumed, 250 c.c.). *Ethylhexylaminomethyl-6-methoxy-4-quinolylcarbinol dipicrate*, isolated from the reduction mixture in 20% yield, had m. p. 170° and was soluble in hot ethyl alcohol (Found: C, 48·8; H, 4·5; N, 14·6. $C_{20}H_{30}O_2N_2,2C_6H_3O_7N_3$ requires C, 48·8; H, 4·6; N, 14·2%). Methyl-6-methoxy-4-quinolylcarbinol picrate was isolated as a by-product and its formation in quantity accounts for the low yield of the desired base.

Propylhexylaminomethyl-6-methoxy-4-quinolylcarbinol.—The product obtained from the appropriate bromomethyl ketone hydrobromide (7·1 g.) and propylhexylamine (8·5 g.) was reduced in the usual way. The resultant oil (5·8 g.) had to be fractionated by the method of differing basicities (cf. King and Ware, J., 1941, 331) before any crystalline derivatives could be obtained. The required *dipicrate*, m. p. 169°, was crystallised from acetone–ethyl alcohol; yield, 17% (Found: C, 49·8; H, 5·1. $C_{21}H_{32}O_2N_2,2C_6H_3O_7N_3$ requires C, 49·5; H, 4·8%).

Methylnonylaminomethyl-6-methoxy-4-quinolylcarbinol.—Condensation of methylnonylamine (4·0 g.) with 6-methoxy-4-quinolyl bromomethyl ketone hydrobromide (3·07 g.), reduction, and fractionation of the product gave an oil (1·06 g.), which was separated by fractional crystallisation into methylmethoxyquinolylcarbinol picrate and *methylnonylaminomethyl-6-methoxy-4-quinolylcarbinol dipicrate*, m. p. 151°, readily soluble in hot ethyl alcohol (Found: C, 50·9; H, 5·2; N, 13·4. $C_{22}H_{34}O_2N_2,2C_6H_3O_7N_3$ requires C, 50·0; H, 4·9; N, 13·7%).

Butylhexylaminomethyl-6-methoxy-4-quinolylcarbinol.—The condensation with butylhexylamine (9·4 g.) and subsequent reduction were carried out as described for the lower homologue. The *dipicrate* (0·7 g.), m. p. 158—159°, was sparingly soluble in ethyl alcohol (Found: C, 50·8; H, 5·2; N, 14·5. $C_{22}H_{34}O_2N_2,2C_6H_3O_7N_3$ requires C, 50·0; H, 4·9; N, 13·7%).

2′ : 2′ : 6′-Trimethylpiperidinomethyl-6-methoxy-4-quinolylcarbinol.—2 : 2 : 6-Trimethylpiperidine (6·0 g.) was condensed with the bromoketone hydrobromide (5·73 g.), and the product reduced (hydrogen consumed, 340 c.c.). From the ether-soluble fraction of the product a crystalline hydrochloride (0·7 g.), m. p. 214—218° (decomp.), was isolated by fractional crystallisation from ethyl alcohol. Although apparently homogeneous, this hydrochloride did not give satisfactory analytical figures. It was converted into the *dipicrate*, m.p. 214°, which was crystallised repeatedly from acetone containing 5% of free picric acid (Found: C, 49·4; H, 3·8. $C_{20}H_{28}O_2N_2,2C_6H_3O_7N_3$ requires C, 48·9; H, 4·3%).

Attempts to obtain morpholinomethyl-6-methoxy-4-quinolylcarbinol were unsuccessful, reduction always leading to formation of methyl-6-methoxy-4-quinolylcarbinol.

We are grateful to Miss Ann Bishop, D.Sc., working in the laboratory of Prof. Keilin, F.R.S., at the Molteno Institute, Cambridge, for the biological results. To Mr. E. V. Wright and Mr. N. Schunmann we are indebted for technical assistance.

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79. *Antiplasmodial Action and Chemical Constitution. Part VI. Compounds related to Lepidylamine.*

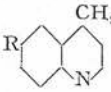
By Thomas S. Work.

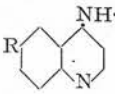
The aim of this investigation was the preparation of polyamines containing the lepidylamine nucleus for test as antimalarials. Polyamines have been synthesised containing the diethylamino*iso*amyl side chain of plasmochin attached to the lepidylamine nucleus and to 6-chloro- and 6-methoxy-lepidylamine. Reduction of amidodichlorides by stannous chloride in ethereal hydrogen chloride (Work, following paper) is a valuable preparative method for the syntheses of substituted lepidylamines from cinchoninamides.

Sulphanilyl derivatives of the afore-mentioned lepidylamines have also been prepared.

In Part IV (Work, J., 1940, 1315) the conclusion was reached that, for the exhibition of antiplasmodial action in polyamines, the presence of the quinoline nucleus was of major importance. A series of bases of the

type (I) has therefore been prepared, allied to compounds of type (II) reported by Magidson and Rubtzow (*J. Gen. Chem., U.S.S.R.*, 1937, **17**, 1896) to be active.

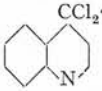
(I.) 

(II.) 

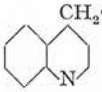
α-Diethylamino-δ-aminopentane, present in plasmochin and atebrin and reported by Fulton (*Ann. Trop. Med. Parasit.*, 1940, **34**, 53) to have slight antiplasmodial activity, was considered the most desirable type of side chain to introduce into bases of type (I).

α-Diethylamino-δ-aminopentane was condensed with benzaldehyde, salicylaldehyde, anisaldehyde and *m*-nitrobenzaldehyde; the azomethine, obtained in good yield, was readily reduced to the desired amine with either palladium–charcoal or palladised strontium carbonate catalyst. In the case of the nitro-compound simultaneous reduction to the amino-group took place. Similarly quinoline-4-aldehyde (kindly supplied by Prof. Clemo) condensed with α-diethylamino-δ-aminopentane at room temperature to give an oily azomethine which was readily reduced to α-diethylamino-δ-amyl-lepidylamine (V). Quinoline-4-aldehyde is not, however, very readily prepared (Clemo and Hoggarth, J., 1939, 1242; cf. Kaplan, *J. Amer. Chem. Soc.*, 1941, **63**, 2654) and substituted quinoline-4-aldehydes are unknown, so an easier route to the desired quinolinepolyamines was sought, and found in the reduction of the cinchoninamide of α-diethylamino-δ-aminopentane (III) to the base (V) by the action of stannous chloride on the amidodichloride (IV), a new type of reaction which is discussed elsewhere (Work, *loc. cit.*).

(III.)



(IV.)

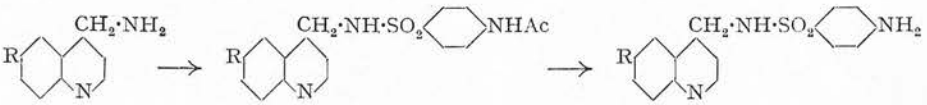


(V.)

This reaction provided an easy route for the subsequent analogous syntheses of α-diethylamino-δ-amyl-6-methoxylepidylamine and *α-diethylamino-δ-amyl-6-chlorolepidylamine*. 6-Chlorolepidylamine was synthesised from 5-chloroisatin, which was converted into *6-chlorocinchoninic acid* by condensation with pyruvic acid, followed by partial decarboxylation of the resulting *6-chloroquinoline-2 : 4-dicarboxylic acid* (cf. Renshaw and Friedman, *J. Amer. Chem. Soc.*, 1939, **61**, 3320). 6-Chlorocinchoninic acid was converted into the *amide*, which was readily dehydrated in 77% yield by boiling with phosphoric oxide in nitrobenzene, a procedure found to be much more satisfactory in this case and also in the preparation of 4-cyanoquinoline and 6-methoxy-4-cyanoquinoline, than the thionyl chloride method described by Wojahn (*Arch. Pharm.*, 1936, **274**, 83).

Attempts to prepare dialkylaminoalkylaminolepidines by the condensation of *diethylamino-ω-chlorohexane* with lepidylamine were unsuccessful.

The observation that derivatives of sulphanilamide or diaminodiphenylsulphone have some action on experimental malaria (Coggeshall, *J. Exp. Med.*, 1940, **71**, 13) and on human malaria (Coggeshall, Maier, and Best, *J. Amer. Med. Assoc.*, 1941, **117**, 1077) suggested the preparation of sulphonamide derivatives of lepidylamine for trial on bird malaria. Condensation of acetylsulphanilyl chloride with lepidylamine and its 6-chloro- and 6-methoxy-derivatives proceeded without difficulty and the N^4-acetylsulphonamides were hydrolysed by alkali to the corresponding aminosulphonamides.



None of the polyamines containing the quinoline nucleus and none of the sulphonamides showed any activity against *Plasmodium relictum* in canaries. The sulphonamides were highly toxic and are being tested against other organisms.

Experimental.

α-Diethylamino-δ-amylbenzylamine.—When the initial reaction between benzaldehyde (2·1 g.) and diethyl-δ-aminoamylamine (3·1 g.) had subsided, the product was heated for 2 minutes on the steam-bath, dissolved in absolute alcohol (50 c.c.), and reduced with a palladium–charcoal catalyst. Alcohol was removed, and the product distilled; the fraction (3·06 g.), b. p. 184—188°/25 mm., had b. p. 187—189°/25 mm. on redistillation (Found: C, 76·6; H, 11·2; N, 11·9. $C_{16}H_{28}N_2$ requires C, 77·4; H, 11·3; N, 11·9%).

*α-Diethylamino-δ-amyl-*p*-methoxybenzylamine*, obtained from anisaldehyde (2·72 g.) and diethyl-δ-aminoamylamine (3·16 g.) by the above procedure, had b. p. 218°/17 mm. (4·73 g.) (Found: C, 73·0; H, 10·6. $C_{17}H_{30}ON_2$ requires C, 73·4; H, 10·8%).

*α-Diethylamino-δ-amyl-*m*-aminobenzylamine* (2·5 g.), similarly obtained (*m*-nitrobenzaldehyde, 3·02 g.; diethyl-δ-aminoamylamine, 3·16 g.; hydrogen consumed, 1910 c.c.), had b. p. 184—186°/25 mm. (Found: C, 72·8; H, 10·8. $C_{16}H_{29}O_3$ requires C, 73·0; H, 11·0%).

α-Diethylamino-δ-amyl-lepidylamine.—Quinoline-4-aldehyde (0·43 g.) was added to diethyl-δ-aminoamylamine (0·43 g.), and the mixture warmed to complete the reaction. The azomethine was reduced in the usual way, and the oily product purified as the *dipicrate*, which crystallised from alcohol in needles, m. p. 147—148° (Found: C, 49·0; H, 5·0; N, 17·0. $C_{19}H_{29}N_3,2C_6H_3O_7N_3$ requires C, 49·2; H, 4·7; N, 16·7%).

Method II. Cinchoninic acid (2 g.) was converted by thionyl chloride into the acid chloride hydrochloride, which was powdered and added slowly to a solution of diethyl-δ-aminoamylamine (6·0 g.) in chloroform (100 c.c.). The

solution was warmed for a few minutes on the water-bath, washed with water, and dried, and the chloroform and the excess of diethyl-δ-aminoamylamine removed, the latter in a vacuum. The residual viscous amide was dissolved in dry chloroform (25 c.c.), and phosphorus pentachloride (5 g.) added. Chloroform and phosphorus oxychloride were removed and the solid residue was powdered and added to a solution prepared by passing dry hydrogen chloride into a suspension of anhydrous stannous chloride (10 g.) in ether (100 c.c.) until the chloride dissolved and formed a clear lower layer. After 24 hours' shaking, the ether was decanted, and the semi-crystalline sludge dissolved in water, washed with ether, and mixed with a considerable excess of 50% sodium hydroxide solution. Ether then extracted an oil (2·73 g.), which was distilled, giving (1) 1·3 g., b.p. 180—200°/1 mm., and (2) 0·8 g., b. p. 200—220°/1 mm. Fraction (1) gave a dipicrate, m. p. 145—146°, identical with that described above.

Cinchoninonitrile.—Phosphoric oxide (2·0 g.) was added to a boiling solution of cinchoninamide (2·0 g.) in nitrobenzene (15 c.c.). After refluxing for 5 minutes, the solution was poured into water, the residual gum dissolved in hot water and added to the main bulk, sodium hydroxide added in excess, and the nitrobenzene separated and washed. The alkaline solution was extracted with chloroform, the extract added to the nitrobenzene, the chloroform evaporated, and the nitrobenzene diluted with petrol and extracted with 2N-hydrochloric acid. The acid solution was washed with petrol, made alkaline, and extracted with chloroform, which removed almost pure cinchoninonitrile; after crystallisation from petrol the yield was 78%.

Lepidylamine.—This was obtained in almost quantitative yield by reducing 4-cyanoquinoline (2·0 g.) in a mixture of methanol (60 c.c.), N-hydrochloric acid (60 c.c.), and platinic oxide (0·05 g.) (cf. Rabe, *Ber.*, 1913, **46**, 1025).

N^1-*Lepidylsulphanilamide.*—Acetylsulphanilyl chloride (2·2 g.) was added to hot acetone–water (1 : 1; 50 c.c.) containing lepidylamine (1·58 g.) and sodium bicarbonate (0·9 g.), maintained at 68° for ½ hour, and cooled. The crystalline product (2·45 g.), recrystallised from alcohol–water, formed long needles, m. p. 134—136°, and 185—190° after drying at 120°. N^4-Acetyl-N^1-lepidylsulphanilamide (1·0 g.) was refluxed in 10% sodium hydroxide solution (10 c.c.) for 1 hour, and N^1-*lepidylsulphanilamide* (0·68 g.) isolated from the acidified solution by means of sodium bicarbonate; crystallised from methanol, it melted at 194° (Found: C, 61·4; H, 4·7; N, 13·4. $C_{16}H_{15}O_2N_3S$ requires C, 61·4; H, 4·8; N, 13·4%).

Quininamide.—A solution of methyl quininate (45 g., prepared by the same procedure as methyl 6-chlorocinchoninate; see below) in methyl alcohol (280 c.c.) saturated with ammonia was kept for 48 hours at 37° and then concentrated. The quininamide (35 g.) obtained crystallised from ethyl acetate in small hard prisms and from alcohol–water in long needles, both m. p. 210—212° (Found: C, 65·5; H, 4·8. Calc. for $C_{11}H_{10}O_2N_2$: C, 65·3; H, 4·9%). Hirsch (*Monatsh.*, 1896, **17**, 331) gives m. p. 197° (from ethyl acetate).

Quininonitrile.—Quininamide (5 g.) in boiling nitrobenzene (50 c.c.) was treated with phosphoric oxide (7·5 g.), added during 5 mins. After 15 mins.' boiling, the nitrile was isolated (see cinchoninonitrile) and crystallised from alcohol–water; m. p. 155°. Kaufmann and Peyer (*Ber.*, 1912, **45**, 1807) give m. p. 157°.

6-*Methoxylepidylamine.*—A solution of quininonitrile (4·0 g.) in absolute alcohol (700 c.c.) and *N*-hydrochloric acid (120 c.c.) was readily reduced with platinic oxide as catalyst. The catalyst was removed, and the solution of dihydrochloride concentrated to a gum, which crystallised on trituration with alcohol. The product, recrystallised from alcohol–water, had m. p. 255—256° (cf. D.R.-P. 279,193); yield, almost quantitative. The base, obtained from the dihydrochloride by continuous extraction with ether from sodium carbonate solution, was a colourless oil turning violet in air.

N^4-*Acetyl*-N^1-(6-*methoxylepidyl*)*sulphanilamide* (2·2 g.), obtained from acetylsulphanilyl chloride (2·2 g.) and 6-methoxylepidylamine (1·88 g.) by the procedure already described and recrystallised from acetone, had m. p. 215° (Found: C, 59·0; H, 4·7; N, 10·5. $C_{19}H_{19}O_4N_3S$ requires C, 59·2; H, 4·9; N, 10·9%).

N^1-(6-*Methoxylepidyl*)*sulphanilamide*, prepared by refluxing the preceding acetyl derivative with 2N-sodium hydroxide for 45 minutes, and isolated by adding sodium bicarbonate to the acidified solution, was crystallised from acetone; m. p. 194° (Found: C, 59·4; H, 4·9. $C_{17}H_{17}O_3N_3S$ requires C, 59·5; H, 4·9%).

α-*Diethylamino*-δ-*amyl*-6-*methoxylepidylamine.*—Quininic acid (2 g.) was converted by thionyl chloride into the acid chloride hydrochloride. The subsequent procedure was that described for the preparation of α-diethylamino-δ-amyl-lepidylamine (quantities used: diethyl-δ-aminoamylamine, 6 g., in chloroform, 100 c.c.; resulting gum, 3·7 g., in chloroform, 25 c.c.; phosphorus pentachloride, 4·4 g.; anhydrous stannous chloride, 10 g., in ethereal hydrogen chloride, 100 c.c.; 48 hours' shaking with glass beads). The gum obtained from the sludge was distilled at 1·0 mm., fraction (1), b. p. 200—210°, and (2) b. p. 210—212°. Fraction (2) gave a *tripicrate*, m. p. 87—88°, from alcohol (1·36 g.). Fraction (1) gave, after some manipulation, a less pure sample (0·85 g.) of the same picrate (Found: C, 44·0; H, 4·4; N, 15·6. $C_{20}H_{31}ON_3,3C_6H_3O_7N_3$ requires C, 44·8; H, 4·0; N, 16·3%).

6-*Diethylaminohexanol.*—Hexamethylene chlorohydrin (64 g.) (Bennett and Turner, J., 1938, 814) and diethylamine (140 g.) were heated in a sealed bottle at 100° for 16 hours. The excess of diethylamine was recovered, the residue diluted with ether, diethylamine hydrochloride collected, and the ether removed. The resulting oil, distilled at 2 mm., was separated into three fractions boiling at (*a*) 83° (2·5 g.), (*b*) 83—93° (5·0 g.), and (*c*) 93—100° (52·4 g.). Fraction (*c*) redistilled at 2 mm. mainly at 96—99° (47·4 g.) (Found: C, 69·2; H, 13·2. $C_{10}H_{23}ON$ requires C, 69·4; H, 13·3%).

Diethylamino-ω-*chlorohexane.*—Diethylaminohexanol (10·5 g.) in dry chloroform (50 c.c.) was run slowly into thionyl chloride (45 g.) in chloroform (50 c.c.) at 0°. After 1½ hours chloroform and the excess of thionyl chloride were removed under reduced pressure, and alcohol (5 c.c.) added. The product was diluted with ether, washed with sodium carbonate and water, and distilled, giving a fraction (5·76 g.), b. p. 118—120°/19 mm. (Found: C, 62·3; H, 11·6. $C_{10}H_{22}NCl$ requires C, 62·6; H, 11·5%).

5-*Chloroisatin.*—This was prepared from *p*-chloroaniline by a process similar to that described in *Organic Syntheses* (Coll. Vol. I, 321; cf. Sandmeyer, *Helv. Chim. Acta*, 1919, **2**, 238) for the preparation of isatin. A large excess of sodium sulphate in the initial condensation between *p*-chloroaniline, chloral hydrate, and hydroxylamine was essential. The resulting *p*-chloro*iso*nitrosoacetanilide was converted into the isatin by addition to concentrated sulphuric acid at 90—95°, and the temperature was raised to 105° for 10 minutes to complete the reaction (yield, 55 g. from 64 g. of *p*-chloroaniline).

6-*Chloroquinoline*-2 : 4-*dicarboxylic Acid.*—5-Chloroisatin (134 g.) was dissolved in hot 33% aqueous potassium hydroxide (1085 c.c.), and pyruvic acid (114 g.) added (cooling in tap-water). After 48 hours at 37° the mixture was cooled, and the potassium salt collected, washed with a little ice-cold 33% potassium hydroxide solution and with absolute alcohol, and dissolved in water. The 6-*chloroquinoline*-2 : 4-*dicarboxylic acid* was precipitated with hydrochloric acid, redissolved in bicarbonate, and reprecipitated by acid; m. p. about 250° (decomp.) (Found: C, 49·3; H, 3·0; N, 4·9. $C_{11}H_6O_2NCl,H_2O$ requires C, 49·0; H, 3·0; N, 5·2%).

6-*Chlorocinchoninic Acid.*—A suspension of 6-chloroquinoline-2 : 4-dicarboxylic acid (15·5 g.) in dry nitrobenzene (100 c.c.) was boiled for 20 minutes and cooled. The crystalline product (12·25 g.) was very slightly soluble in all organic solvents, but could be recrystallised from nitrobenzene; m. p. 302° (Found: C, 57·9; H, 3·1. $C_{10}H_6O_2NCl$ requires C, 57·9; H, 3·1%).

Methyl ester. The acid (30 g.) was refluxed in dry chloroform (50 c.c.) with thionyl chloride (50 c.c.) for ½ hour,

the excess of chloride removed on the water-bath, methyl alcohol added to the residue, and the ester hydrochloride diluted with water and made alkaline. The *methyl* ester, extracted with chloroform, crystallised from ligroin–benzene in needles (26·0 g.), m. p. 79·5° (Found : C, 59·8; H, 3·6. $C_{11}H_8O_2NCl$ requires C, 59·6; H, 3·6%).

6-*Chlorocinchoninamide*.—The methyl ester was dissolved in excess of methyl alcohol saturated with ammonia and left for 3 days at 37°; the solution was concentrated on the water-bath. The crystalline *amide* (20·85 g.) had m. p. 244° (Found : C, 57·7; H, 3·5. $C_{10}H_7ON_2Cl$ requires C, 58·1; H, 3·4%).

6-*Chlorocinchoninonitrile*.—To a boiling solution of 6-chlorocinchoninamide (7·7 g.) in nitrobenzene (50 c.c.), phosphoric oxide (8·0 g.) was added in several small lots. After 4 mins.' boiling, the hot product was poured into water, and the residual gum dissolved in boiling water and added to the main bulk of solution, which was then made alkaline and diluted with chloroform. The chloroform–nitrobenzene was separated and dried, chloroform removed, and as much as possible of the nitrobenzene distilled under reduced pressure. The residue was triturated with petrol, and the crystalline product (5·53 g.), m. p. 164°, collected. 6-*Chlorocinchoninonitrile* crystallised in long needles, m. p. 164°, from ethyl acetate (Found : C, 63·8; H, 2·9. $C_{10}H_5N_2Cl$ requires C, 63·7; H, 2·7%).

6-*Chloro*-4-*aminomethylquinoline*.—To a solution of the preceding nitrile (4 g.) in absolute alcohol (700 c.c.), N-hydrochloric acid (120 c.c.) and platinic oxide (0·2 g.) were added. The solution, shaken at normal pressure, absorbed 1400 c.c. of hydrogen in 8 hours. On concentration 6-*chloro*-4-*aminomethylquinoline dihydrochloride* (32 g.) separated in rhombs, m. p. about 250° (decomp.), sparingly soluble in alcohol (Found : C, 45·6; H, 4·3; N, 10·5. $C_{10}H_9N_2Cl,2HCl$ requires C, 45·2; H, 4·14; N, 10·5%). The base, obtained from the dihydrochloride and sodium carbonate solution by continuous extraction with ether, was moderately easily soluble in water; it crystallised from ether in colourless needles, m. p. 90°, which turned bright violet on exposure to air.

N^4-*Acetyl*-N^1-(6-*chlorolepidyl*)*sulphanilamide*—6-Chlorolepidylamine (1·93 g.) was dissolved in aqueous acetone (50 c.c.) containing sodium bicarbonate (0·9 g.) and treated with acetylsulphanilyl chloride (2·2 g.) by the same procedure as used previously. The product (2·85 g.) crystallised from acetone in needles (2·75 g.), m. p. 194° (Found : C, 55·4; H, 4·2. $C_{18}H_{16}O_3N_3ClS$ requires C, 55·4; H, 4·2%).

N^1-(6-*Chlorolepidyl*)*sulphanilamide*.—The acetyl derivative (1·73 g.) was refluxed in 2N-sodium hydroxide (15 c.c.) for 1 hour. The product was isolated in the usual way (1·43 g.) and crystallised from methanol; yield 1·05 g., m. p. 200° (Found : C, 55·0; H, 4·1; N, 11·6. $C_{16}H_{14}O_2N_3ClS$ requires C, 55·2; H, 4·0; N, 12·1%).

6-*Chlorocinchoninamide* of *Diethyl*-δ-*aminoamylamine*.—6-Chlorocinchoninic acid (2·0 g) was converted by thionyl chloride into the acid chloride hydrochloride, which was powdered and added to a solution of diethyl-δ-aminoamylamine (6·0 g.) in chloroform (100 c.c.). After ½ hour the chloroform solution was washed with water, dried, and evaporated. The residual brown oil crystallised on prolonged standing and was recrystallised from ligroin (2·65 g.). The *amide*, even when pure, m. p. 99°, could only be crystallised with difficulty, as, when dissolved in hot ligroin, it separated as a stiff gel which only crystallised very slowly (Found : C, 65·5; H, 7·4; N, 11·7. $C_{19}H_{26}ON_3Cl$ requires C, 65·6; H, 7·5; N, 12·1%).

α-*Diethylamino*-δ-*amyl*-6-*chlorolepidylamine*.—The preceding amide (2·4 g.) was dissolved in dry chloroform (20 c.c.) and treated with phosphorus pentachloride (2·9 g.). Chloroform was distilled off on the steam-bath, and the residue heated at 100° for ½ hour. A solution of stannous chloride (10 g.) in ethereal hydrogen chloride (100 c.c.) was added to the cooled residue, which was then shaken for 48 hours with glass beads. The polyamine (2·14 g.) was isolated by the usual procedure as a brown oil and converted into a picrate (tripicrate?), m. p. 97—99°, which crystallised readily from acetone–alcohol. The picrate was reconverted into the base, which was distilled in a high vacuum before analysis (Found : C, 67·8; H, 8·6; N, 13·1; Cl, 10·8. $C_{19}H_{28}N_3Cl$ requires C, 68·3; H, 8·5; N, 12·6; Cl, 10·6%).

I wish to thank Miss Ann Bishop, D.Sc., of the Molteno Institute, Cambridge, for carrying out biological tests and Mr. N. Schunmann for technical assistance. Acknowledgment is also made to Mr. Barrowcliff of Imperial Chemical Industries Ltd. for a gift of diethylamino*iso*amylamine.

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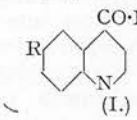
80. *The Synthesis of Amines from Amides through the Amidodichlorides.*

By Thomas S. Work.

The Sonn–Müller reaction for the preparation of aldehydes from anils, by reduction of the imidochlorides by stannous chloride and ethereal hydrogen chloride, is not applicable to quinoline-4-carboxyamides. The reaction of certain quinoline-4-carboxyamides with phosphorus pentachloride gives amidodichlorides which are reduced under the above conditions to secondary amines. The mechanism of both types of reaction is discussed. An analogous type of reaction is possible with *quinoline-4-aldehyde anil*, which gives with stannous chloride and ethereal hydrogen chloride *N-phenyl-lepidylamine*. Application of the Sonn–Müller conditions to *nicotinethylamide* gives pyridine-3-aldehyde and 3-*N-ethylaminomethylpyridine*, through the operation of both types of reaction.

It being desired (Work, preceding paper) to have a convenient method for the preparation of quinoline-4-aldehydes, an attempt was made to apply the reaction of Sonn and Müller (*Ber.*, 1919, **52**, 1927), *viz.*, $R{\cdot}CO{\cdot}NHR' \longrightarrow R{\cdot}CCl{:}NR' \longrightarrow R{\cdot}CH{:}NR' \longrightarrow R{\cdot}CHO + NH_2R'$. Accordingly *cinchoninanilide* (I; R = H, R′ = Ph) was allowed to react in toluene with phosphorus pentachloride (1 mol.) and the product (imidochloride?) was treated with stannous chloride in ethereal hydrogen chloride as recommended by Sonn and Müller. The product was, however, not quinoline-4-aldehyde, as might be expected, but *N*-phenyl-lepidylamine (II; R = H, R′ = Ph).



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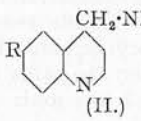

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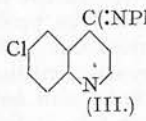


(III.)

With phosphorus pentachloride (2 mols.) *N*-phenyl-lepidylamine was obtained in 68% yield. *Cinchoninomethylamide* (I; R = H, R′ = Me) and 6-*chlorocinchoninanilide* (I; R = Cl; R′ = Ph), treated in chloroform

with phosphorus pentachloride, the products being reduced with stannous chloride, gave N-*methyl-lepidylamine dihydrochloride* (II + 2HCl; R = H, R′ = Me) and N-*phenyl-6-chlorolepidylamine* (II; R = Cl, R′ = Ph) respectively in good yield. 6-Methoxycinchoninamide (I; R = OMe, R′ = H) failed to furnish any amine. Cinchoninodiethylamide was recovered unchanged even after heating at 150° with phosphorus pentachloride. This result was not wholly unexpected in view of von Braun's work (*Ber.*, 1904, **37**, 2812, 2915) on the action of phosphorus pentachloride on the amides of secondary amines: $R{\cdot}CO{\cdot}NR'R'' \longrightarrow R{\cdot}CCl{:}NR' + R''Cl \longrightarrow R{\cdot}CO{\cdot}NHR'$.

Wallach (*Annalen*, 1877, **184**, 4) found that phosphorus pentachloride and acid amides gave in general amidodichlorides which lost hydrogen chloride spontaneously with formation of imidochlorides; from ethyl oxamate, however, he obtained an unstable crystalline amidodichloride.

Lander and Laws (J., 1904, **85**, 1695) found that hydrogen iodide combined with benzanilide imidochloride to give an amidochlorideiodide, Ph·CClI·NHPh, and Stephen and Bleloch (J., 1931, 888) showed that benzanilide imidochloride combined with hydrogen chloride in ethereal solution to give a crystalline amidodichloride hydrochloride. Hallmann (*Ber.*, 1876, **9**, 846) also isolated a crystalline amidodichloride, $Ph{\cdot}CCl_2{\cdot}NMe_2$, by the action of carbonyl chloride on benzodimethylamide.

It thus seemed probable that the production of secondary amines under the Sonn–Müller conditions in the quinoline series was dependent on the intermediate formation of amidodichlorides, which, under suitably controlled conditions, might be reduced to amines by stannous chloride. However, when the product of the reaction of benzanilide with phosphorus pentachloride at 0° was reduced with stannous chloride at 0°, only benzaldehyde and aniline were formed. Stephen and Bleloch's benzanilide amidodichloride hydrochloride, similarly reduced at 0°, also gave only benzaldehyde and aniline. The amidodichlorides of ethyl oxamate and ethyl ethyloxamate could not be reduced to the corresponding amines.

In view of these failures a more detailed study was undertaken of the reaction in the quinoline series and 6-chlorocinchoninanilide (I; R = Cl, R′ = Ph) was chosen as likely to provide the most readily crystallisable intermediates. When 6-chlorocinchonanilide in chloroform was treated with phosphorus pentachloride (2 mols.), 6-*chlorocinchoninanilide hydrochloride* corresponding to slightly more than one half the starting material separated almost immediately. The chloroform-soluble material was an unstable yellow oil, which gave on boiling with aniline NN-*diphenyl-6-chloro-4-quinolylamidine* (III) or with stannous chloride and hydrogen chloride *N*-phenyl-6-chlorolepidylamine (II; R = Cl, R′ = Ph). If the original mixture of anilide and pentachloride was refluxed for an hour, the only product was a yellow oil which could be reduced to *N*-phenyl-6-chlorolepidylamine. This oil, also obtained from solid 6-chlorocinchoninanilide hydrochloride in chloroform and phosphorus pentachloride, was eventually crystallised from carbon disulphide. It appears to have the formula $C_{16}H_{11}N_2Cl_3$. The solubility of this compound in chloroform, contrasted with the insolubility of cinchoninanilide hydrochloride, precluded the possibility that it was the imidochloride hydrochloride and it is justifiably formulated as 6-*chlorocinchoninanilide amidodichloride*, a structure consistent with the formation of the amidine and *N*-phenyl-6-chlorolepidylamine mentioned above. The formation of 6-chlorocinchoninanilide hydrochloride as one of the primary products of the reaction between the anilide and phosphorus pentachloride is consistent with the interpretation of the preliminary phase of the reaction as involving enolisation of the amide:

$$\begin{matrix} R{\cdot}CO{\cdot}NHR' \\ \Updownarrow \\ R{\cdot}C(OH){:}NR' \end{matrix} + PCl_5 \longrightarrow R{\cdot}CCl{:}NR' + R{\cdot}CO{\cdot}NHR',HCl + POCl_3$$

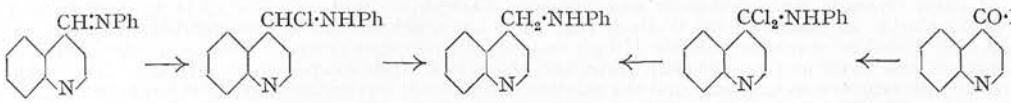
This interpretation is supported by the failure of disubstituted amides to undergo a similar reaction.

Since, however, iminochlorides add on hydrogen chloride under anhydrous conditions and since the Sonn-Müller reaction is carried out in the presence of excess of ethereal hydrogen chloride, it seems probable that the substance undergoing reduction with stannous chloride is always the amidodichloride. This being so, an explanation is required of why, in the case of amides derived from quinoline-4-carboxylic acids and primary amines, reduction to the secondary amine takes place, but in the case of benzanilide and of other anilides studied by Sonn and Müller reduction to an anil is the course followed, with subsequent hydrolysis to the aldehyde.

Hantzsch and Schwab (*Ber.*, 1901, **34**, 822) formulated the hydrogen chloride addition products of anils as true salts, $R{\cdot}CH{:}\overset{+}{N}HR'\overset{-}{]}Cl$, although in certain cases sodium carbonate solution converted them into aldehydeanilines, R·CH(OH)·NHR′. Franzen and Henglein (*J. pr. Chem.*, 1915, **91**, 245), however, from observations on the addition of bromine to anils, formulated the hydrobromides of anils as R·CHBr·NHR′. This view was disputed by Hantzsch (*Ber.*, 1915, **48**, 1340). In accordance with modern theories the hydrogen chloride addition products of anils can probably be best regarded as mesomeric between the ionic and the covalent state. In the case of the amidodichlorides similar mesomerism is probable and in cases where secondary amines are produced by stannous chloride reduction, it is reasonable to assume that the electronic rearrangement due to the adjacent heterocyclic ring (R) is such that the halogen atoms become attached to carbon by covalent links, $R{\cdot}CCl_2{\cdot}NHR'$; on the other hand, in compounds where reduction to an anil takes place the linkage of halogen must approach the ionic state. The general scheme to cover both types of product can be formulated thus:

$$R{\cdot}CH_2{\cdot}NHR' \longleftarrow R{\cdot}CCl_2{\cdot}NHR' \rightleftarrows R{\cdot}CCl{:}NHR'^{+}Cl^{-} \longrightarrow R{\cdot}CH{:}NHR'^{+}Cl^{-} \longrightarrow R{\cdot}CHO + R'NH_3^{+}Cl^{-}$$

If this view is correct, it would seem to follow that an anil prepared from quinoline-4-aldehyde should combine with hydrogen chloride to give a covalent addition product reducible to a secondary amine. This proved to be so, for quinoline-4-aldehyde anil treated with stannous chloride in ethereal hydrogen chloride solution gave a secondary amine identical with that obtained from cinchoninanilide.



When, however, the same reaction was applied to benzaldehyde-anil, benzaldehyde was isolated in quantitative yield.

An insufficient number of examples have been studied up to the present to determine what structural factors govern the direction of reduction of the amidodichlorides; it has been found, however, that when nicotinethylamide was treated with phosphorus pentachloride and the product submitted to the Sonn–Müller conditions, it gave, mainly, the anil which decomposed to yield pyridine-3-aldehyde, identified as its phenylhydrazone. 3-Ethylaminomethylpyridine was a minor product and was identified as the *picrate* and *platinichloride*. It is evident that in this case the behaviour is intermediate between that shown by 4-substituted quinolines and that shown by benzanilide in that both types of reaction have taken place, but in unequal proportion.

Experimental.

Cinchoninanilide.—Cinchoninic acid (20 g.) was converted by thionyl chloride into the acid chloride hydrochloride, which was added in small lots to a solution of aniline (40 g.) in chloroform (200 c.c.). Chloroform was removed, the residue suspended in water, and the *anilide* collected, twice precipitated from acetone solution by an equal volume of water, and crystallised from benzene; it formed stout needles (17 g.), m. p. 161—162° (Found: C, 76·9; H, 4·7. $C_{16}H_{12}ON_2$ requires C, 77·4; H, 4·8%).

N-Phenyl-lepidylamine.—Cinchoninanilide (12·4 g.) was boiled in toluene with phosphorus pentachloride (11·0 g.) for 3 hours, the toluene distilled, and the solid residue added in small portions to a solution of anhydrous stannous chloride (40 g.) in dry ethereal hydrogen chloride (200 c.c. saturated with hydrogen chloride). After 24 hours' shaking with glass beads, the ether was decanted, the sludge washed with ether and boiled with 5N-hydrochloric acid (600 c.c.) until most of the solid dissolved, and the solution filtered hot. The tin salt (18 g.) that separated on cooling was suspended in water, and sodium hydroxide (50% solution) added in considerable excess. Ether extracted a crystalline substance, which was triturated with light petroleum (yield, 7·22 g.) and fractionally crystallised from methyl alcohol–water, giving cinchoninanilide (0·35 g.) and mainly *N*-phenyl-lepidylamine, m. p. 121°.

When 2 mols. of phosphorus pentachloride were used, cinchoninanilide (1·24 g.) gave *N-phenyl-lepidylamine* (0·8 g.) as the sole product (Found: C, 82·0; H, 6·0; N, 12·0. $C_{16}H_{14}N_2$ requires C, 82·0; H, 6·0; N, 12·0%).

Cinchoninomethylamide.—Methyl cinchoninate (2·0 g.) and 33% alcoholic methylamine (4·5 c.c.) were heated at 100° in a sealed tube for 6 hours, the alcohol removed, and the *amide* (1·7 g.) crystallised from ether; m. p. 111° (Found: C, 70·4; H, 5·4. $C_{11}H_{10}ON_2$ requires C, 70·9; H, 5·4%).

N-Methyl-lepidylamine.—Cinchoninomethylamide (1·5 g.) in dry chloroform (10 c.c.) was refluxed with phosphorus pentachloride (2·5 g.; 1½ mols.) for ½ hour, chloroform and phosphorus oxychloride removed in a vacuum, and the product dissolved in dry chloroform (10 c.c.) and added to a solution of stannous chloride (6·5 g.) in ethereal hydrogen chloride (65 c.c.). The mixture was shaken for 24 hours, diluted with water, and washed with ether, sodium hydroxide (50% solution) added in considerable excess, and the chloroform-soluble material extracted from the cooled solution. The extract was a reddish oil which gave a crystalline hydrochloride (1·51 g.) from alcohol, m. p. 215—220° (decomp.); recrystallised from methanol, it formed prisms or needles of *N-methyl-lepidylamine dihydrochloride* (Found for material dried at 100°: C, 53·4; H, 6·0. $C_{11}H_{12}N_2,2HCl$ requires C, 53·9; H, 5·7%). The base was a colourless oil soluble in water and in ether.

Cinchoninodiethylamide.—The acid chloride hydrochloride obtained from cinchoninic acid (3 g.) was suspended in dry benzene, excess of diethylamine added, and after 15 minutes the solution diluted with chloroform and water. The oil (5·5 g.) obtained from the chloroform–benzene was distilled, and the fraction (4·2 g.), b. p. 180°/2 mm., converted into the *picrate*, m. p. 189°, which was crystallised from alcohol (Found: C, 52·6; H, 4·4; N, 15·3. $C_{14}H_{16}ON_2,C_6H_3O_7N_3$ requires C, 52·5; H, 4·2; N, 15·3%).

6-Chlorocinchoninanilide.—This, prepared from 6-chlorocinchoninic acid (Work, *loc. cit.*) (10 g.) (for details, see cinchoninanilide) and crystallised from acetone–water and from acetone (yield, 10·8 g.), had m. p. 205° (Found: C, 68·0; H, 4·0. $C_{16}H_{11}ON_2Cl$ requires C, 67·9; H, 3·9%).

N-Phenyl-6-chlorolepidylamine.—6-Chlorocinchoninanilide (2 g.) in chloroform (50 c.c.) at 50° reacted vigorously with phosphorus pentachloride (2 mols.). The pale yellow solid that separated almost immediately (1·5 g.) was washed with chloroform, dried (Found: C, 59·8; H, 3·8; N, 8·5; Cl, 21·9. $C_{16}H_{11}ON_2Cl,HCl$ requires C, 60·2; H, 3·8; N, 8·8; Cl, 22·2%), and identified as *6-chlorocinchoninanilide hydrochloride* by reconversion into the original material. The chloroform-soluble product was an unstable yellow oil, which crystallised and gave, on boiling with aniline, *NN-diphenyl-6-chloro-4-quinolylamidine* (0·6 g.), sparingly soluble in alcohol, which crystallised from acetone in prisms, m. p. 207° (Found: C, 73·4; H, 4·5; N, 11·9. $C_{22}H_{16}N_3Cl$ requires C, 73·8; H, 4·5; N, 11·9%).

A suspension of 6-chlorocinchoninanilide hydrochloride (0·5 g.) in chloroform (5 c.c.) and phosphorus pentachloride was refluxed for ½ hour, most of the chloroform removed from the clear orange solution, and dry carbon disulphide (15 c.c.) added; the unstable orange needles obtained (0·7 g.) were immediately transferred to a desiccator. (1) A sample, heated with aniline, gave *NN*-diphenyl-6-chloro-4-quinolylamidine. (2) A sample (0·38 g.) was reduced with stannous chloride in ethereal hydrogen chloride. The colourless crystalline product (180 mg.) was *N-phenyl-6-chlorolepidylamine*, m. p. 129°, sparingly soluble in petroleum (Found: C, 72·0; H, 5·0; N, 10·7. $C_{16}H_{13}N_2Cl$ requires C, 71·5; H, 4·8; N, 10·4%). The hydrochloride had m. p. 158—160° (decomp.). The *nitrosoamine* crystallised from ether in needles, m. p. 131° (Found: C, 65·2; H, 4·2; N, 14·1. $C_{16}H_{12}ON_3Cl$ requires C, 64·5; H, 4·0; N, 14·1%). (3) A sample was dissolved in dry chloroform containing a trace of phosphorus pentachloride, the solution evaporated almost to dryness, and carbon disulphide added. The orange-coloured crystals obtained were very unstable and analysis (Found: C, 54·2; H, 3·4; N, 7·6; Cl, 31·6. $C_{16}H_{11}N_2Cl_3$ requires C, 56·9; H, 3·2; N, 8·3; Cl, 31·5%) showed that they consisted mostly of 6-chlorocinchoninanilide amidodichloride.

Quinoline-4-aldehyde Anil.—Quinoline-4-aldehyde (0·3 g.) and aniline (0·18 g.) were heated at 100° for 15 minutes.

The *anil* was dried in a vacuum and crystallised from ether–ligroin, forming thin platelets (0·35 g.), m. p. 85° (Found: C, 82·4; H, 5·3. $C_{16}H_{12}N_2$ requires C, 82·8; H, 5·2%).

Reduction of Quinoline-4-aldehyde Anil.—The anil (0·35 g.) was shaken with stannous chloride (1·5 g.) in ethereal hydrogen chloride (15 c.c.) for 24 hours. The product, isolated by extraction with ether from alkaline solution and crystallised from ligroin, had m. p. 120—121°, not depressed by *N*-phenyl-lepidylamine.

Reaction of Ethyl Oxamate Amidodichloride and Stannous Chloride.—Ethyl oxamate (2·34 g.) was treated with phosphorus pentachloride as described by Wallach (*loc. cit.*); the amidodichloride, precipitated by petroleum, was washed, dried, and added to stannous chloride (10 g.) in ethereal hydrogen chloride (100 c.c.). After 24 hours the product was poured into water and washed with ether, and the acid solution evaporated to dryness. The residue was dissolved in water, tin removed as sulphide, and the solution evaporated, leaving ammonium chloride (0·7 g.).

A similar experiment with ethyl ethyloxamate gave oxalomonoethylamide.

Nicotinethylamide.—Methyl nicotinate (15 g.), heated with 33% alcoholic ethylamine (30 c.c.) in a sealed tube at 100° for 8 hours, gave an oil (13 g.) which distilled at 152°/12 mm., solidified, and could be crystallised from ether; m. p. 57° (Found: C, 63·7; H, 6·6. $C_8H_{10}ON_2$ requires C, 64·0; H, 6·7%).

Reaction of Nicotinethylamide with Phosphorus Pentachloride and Stannous Chloride.—The product of reaction between the ethylamide (3·3 g.) in chloroform (10 c.c.) and phosphorus pentachloride (9·5 g.; 2 mols.) was heated at 120° for ½ hour, phosphorus oxychloride removed in a vacuum, and the residual oil shaken with stannous chloride (14 g.) in ethereal hydrogen chloride (140 c.c.) for 24 hours. Water was then added, the acid solution washed with ether and basified with sodium hydroxide (50% solution) in considerable excess, and ethylamine removed in a vacuum at room temperature. Continuous extraction with ether removed a pale brown oil, which was fractionally distilled, giving (*a*) b. p. 200—220°/760 mm. (0·8 g.), (*b*) b. p. 220—240°/760 mm. (0·22 g.), and (*c*) b. p. 150—180°/14 mm. (0·9 g.). Fraction (*c*) crystallised and was mainly unchanged amide. Fractions (*a*) and (*b*) gave with phenylhydrazine in dilute acetic acid a crystalline phenylhydrazone, m. p. 157° (Found: C, 72·7; H, 5·7. Calc. for $C_{12}H_{11}N_3$: C, 73·1; H, 5·6%). Harries and Lénárt (*Annalen*, 1915, **410**, 115) give m. p. 158° for pyridine-3-aldehyde phenylhydrazone. Fractions (*a*) and (*b*) also gave with platinic chloride and hydrochloric acid the crystalline *platinichloride* of *3-ethylaminomethylpyridine*, sparingly soluble in cold water (Found: N, 4·8; Pt, 35·8. $C_8H_{12}N_2PtCl_6$ requires N, 5·1; Pt, 35·5%).

A sample of fraction (*b*) which appeared to contain a larger proportion of secondary amine than (*a*) was dissolved in methanol, and the aldehyde removed by oxidation with ammoniacal silver nitrate. The stable secondary amine was then isolated and converted into a *picrate*, m. p. 207°, sparingly soluble in ethanol (Found: C, 40·8; H, 3·2. $C_8H_{12}N_2,2C_6H_3O_7N_3$ requires C, 40·4; H, 3·0%).

I wish to thank Dr. H. King, F.R.S., for his helpful interest throughout the course of this work.

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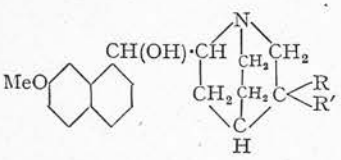
91. *Antiplasmodial Action and Chemical Constitution. Part VII. Derivatives of Quinine and* iso*Quinine.*

By T. S. Work.

Quinine and *β-iso*quinine were ozonised, and the resultant quininal and 3-acetyl-6′-methoxyrubanol reduced catalytically to the corresponding carbinols. Although active, none of the compounds showed antiplasmodial action equal to that of quinine when tested on *Plasmodium relictum* in canaries.

As experiments are in progress in this laboratory designed to synthesise compounds related to quinine, the effect upon antiplasmodial action of minor alterations in the quinine molecule has been investigated.

Seekles (*Rec. Trav. chim.*, **1923**, **42**, 69) prepared quininal (I; R = H, R′ = CHO) by ozonisation of quinine (I; R = H, R′ = CH:CH_2), followed by decomposition of the ozonide with water, but did not test the aldehyde for antiplasmodial properties. Quininol was prepared by Seekles' method and was reduced to quininol (I; R = H, R′ = CH_2·OH) by use of Adams's catalyst. Although quininal proved active in bird malaria, quininol was inactive.



Recently there has been disagreement between Rabe (*Ber.*, 1943, **76**, 320, and earlier papers) and Prelog (*Ber.*, 1941, **74**, 648) over the alleged antiplasmodial properties of synthetic 6′-methoxyrubanol (I, R = R′ = H). It was hoped that β-*iso*quinine, which differs from quinine by having the vinyl side chain in the form :CH·CH_3, on ozonisation might give a ketone which could be reduced to 6′-methoxyrubanol. Unfortunately *iso*quinine ozonide on decomposition with water gave, without loss of carbon atoms, 3-*acetyl*-6′-*methoxyrubanol* (I; R = H, R′ = CO·CH_3), which was reduced to 3-hydroxyethyl-6′-methoxyrubanol, characterised as its crystalline *dihydrobromide*. The alternative procedure of obtaining 6′-methoxyrubanol by decarboxylation of quitenine (I; R = H, R′ = CO_2H) has not so far proved feasible.

The results of tests on bird malaria due to *P. relictum* in canaries are shown in the table:

Substance.	Dose, mg. per 20 g. body weight.	Day of appearance of parasites in blood.	Remarks.
Quinine	6 × 2·5 *	12th—14th	—
Quininal	6 × 2·5	12th—14th	M.T.D.†
	6 × 0·625	8th	—
Quininol	6 × 5	6th	M.T.D.
Acetyl-6′-methoxyrubanol	6 × 10	10th—11th	M.T.D.
Hydroxyethyl-6′-methoxyrubanol	6 × 10	10th—12th	M.T.D.
Controls	—	5th	—

* A dose of 2·5 mg. daily on 6 consecutive days. † M.T.D. = maximum tolerated dose.

3-Hydroxyethyl-6′-methoxyrubanol (α-hydroxydihydroquinine) was previously prepared and fully characterised by Henry, Solomon, and Gibbs (J., 1937, 601), by methylation of α-hydroxydihydro*apo*quinine, and a sample prepared by this method was kindly supplied by Mr. Solomon. The base prepared by reduction of 3-acetyl-6′-methoxyrubanol could not be crystallised even on inoculation with the above α-base, but gave a crystalline *dihydrobromide*, which melted with decomposition at 192—195° and had a specific rotation of $[\alpha]_D^{20°}$ — 142° (*c* = M/80) in N/10-hydrobromic acid. The crystalline base supplied by Mr. Solomon gave a dihydrobromide with similar solubilities and crystalline form, but with a melting point between 198° and 210° and a specific rotation of $[\alpha]_D^{20°}$ — 120° (*c* = M/80). As two asymmetric centres are involved in the formation of these bases, the new base in the form of its dihydrobromide is one of the remaining diastereoisomerides.

The failure of Henry, Solomon, and Gibbs (*loc. cit.*) to obtain more than traces of acetaldehyde on ozonisation of β-*iso*quinine now receives a simple explanation. It is due to the formation of 3-acetyl-6′-methoxyrubanol and this observation does not vitiate, in any way, the structure assigned to β-*iso*quinine.

Experimental.

Quininal.—The method of Seekles (*loc. cit.*) was found to be satisfactory only when extreme precautions were taken to dry the ethyl acetate and petroleum used in the purification of the ozonide.

Quininol.—As there is considerable loss in the purification of quininal, it was found more satisfactory to reduce crude quininal obtained by treatment of the ozonide with water. Purified ozonide (6 g.), decomposed with water and reduced catalytically in ethanol with Adams's catalyst (500 c.c. of hydrogen), gave crystalline quininol dihydrobromide, $[\alpha]_D^{18°}$ — 146° (*c* = M/50, water). The free base could not be crystallised, and the dihydrobromide, although crystalline, gave unsatisfactory analytical results owing to decomposition on drying. However, *quininol monosulphate*, m. p. 149°, crystallised readily from water [Found for material dried at 90° in a vacuum: C, 60·3; H, 6·9; N, 7·2. $(C_{19}H_{24}O_3N_2)_2,H_2SO_4$ requires C, 60·5; H, 6·6; N, 7·4%].

3-*Acetyl*-6′-*methoxyrubanol.*—β-*iso*Quinine (5 g.) was dissolved in dry chloroform (50 c.c.), and dry ozone (3 l. of 13% ozone) bubbled slowly through the solution cooled in ice–salt. Chloroform was removed at reduced pressure without rise in temperature, and 3% acetic acid (50 c.c.) added. After keeping for 48 hours at room temperature, excess of aqueous ammonia was added, and the product extracted with chloroform. The residue after removal of chloroform was separated by fractional crystallisation from acetone into unchanged β-*iso*quinine (1·5 g.), readily soluble, and 3-*acetyl*-6′-*methoxyrubanol* (2·4 g.), needles, m. p. 198—200°, sparingly soluble in acetone (Found: C, 70·5; H, 7·1; N, 8·2. $C_{20}H_{24}O_3N_2$ requires C, 70·6; H, 7·1; N, 8·2%). Acetylmethoxyrubanol dihydrochloride crystallised from alcohol in prisms, but owing to decomposition on drying could not be analysed.

An ethylene oxide formulation of acetylmethoxyrubanol is not impossible, but is considered unlikely, since the compound is stable under conditions which normally rupture the ethylene oxide ring.

3-*Hydroxyethyl*-6′-*methoxyrubanol.*—Acetylmethoxyrubanol (0·5 g.), reduced catalytically in the same way as quininal, gave an oily base. The *dihydrobromide* obtained from the base melted at 192—194° (decomp.) and crystallised in clusters of needles from a mixture of equal parts of alcohol and acetone (Found for material dried at 90°: C, 47·3; H, 5·8; N, 5·3. $C_{20}H_{26}O_3N_2$,2HBr requires C, 47·6; H, 5·6; N, 5·5%).

I wish to thank Dr. T. A. Henry of the Wellcome Foundation for the gift of β-*iso*quinine, Miss Ann Bishop, D.Sc., working in the laboratories of Professor D. Keilin, F.R.S., at the Molteno Institute, Cambridge, for the biological results, and Dr. H. King, F.R.S., for his interest and advice.

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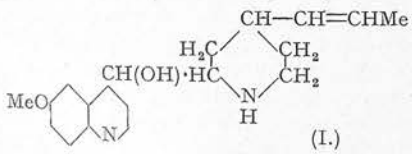
51. *The Synthesis of Antimalarial Compounds Related to Niquidine. Part I. Model Experiments on the Synthesis of Quinolyl Carbinols.*

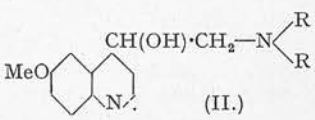
By T. S. Work.

Model experiments to examine the practicability of three alternative methods for the synthesis of compounds of niquidine type are described. Condensation of a quinoline-4-aldehyde with a 3-ketopiperidine without elimination of water was successfully achieved but the keto group could not be reduced. Condensation of an aliphatic aldehyde with a 4-acetylquinoline gave an unsaturated ketone which was reduced to a quinolylalkylcarbinol. Condensation of a quinoline-4-aldehyde with aliphatic nitro-compounds gave nitro-hydroxy-derivatives of quinoline reducible to aminoquinolyl-carbinols. α-Piperidyl-6-methoxy-4-quinolylcarbinol was synthesised by this method.

Niquidine, a degradation product of quinidine, has been reported (Buttle, Henry, Solomon, Trevan and Gibbs, *Biochem. J.*, 1938, **32**, 47) to be more active than quinine in bird malaria. On the basis of degradative experiments, structure (I) was assigned by Gibbs and Henry (*J.*, 1939, 240) to niquidine.



(I.)



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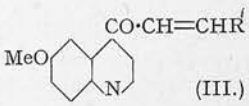
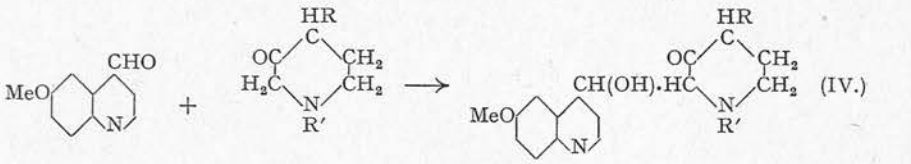


(III.)

Ainley and King (*Proc. Roy. Soc.*, 1938, **125**, B, 60) synthesised α-piperidyl-6-methoxy-4-quinolylcarbinol which may be regarded as niquidine with the unsaturated side-chain removed. This compound was rather less active than quinine in bird malaria. To study the effect of simplifying the " quinuclidine half " of the quinine molecule still further, King and Work (*J.*, 1940, 1307) synthesised a series of 6-methoxy-4-quinolyldialkylaminomethyl-carbinols (II). They found that, in a homologous series (R = methyl to R = heptyl), activity reached a peak at dibutylamino or diamylamino but that the most active member of the series was no more active than α-piperidyl-6-methoxy-4-quinolylcarbinol despite closer similarity to quinine in molecular weight. These results indicated that it might be more profitable to study the synthesis of α-piperidylquinolylcarbinols with suitable alkyl or alkenyl substituents in the piperidine ring.

Clearly such a synthesis might be possible starting from quininic ester and alkylpiperidines using the method of Ainley and King (*loc. cit.*), but as the higher alkylpiperidines are not readily obtainable and as the overall yield by this method was not high it was considered worth while to study alternative methods for linking the quinoline half of the molecule with suitable bases.

Three possible routes have been studied : (i) condensation of 6-methoxy-4-acetoquinoline with aldehydes to give unsaturated ketones; (ii) condensation of 6-methoxyquinoline-4-aldehyde with 3-ketopiperidines; and (iii) condensation of 6-methoxyquinoline-4-aldehyde with suitable nitro compounds.



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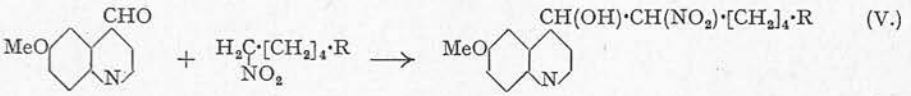
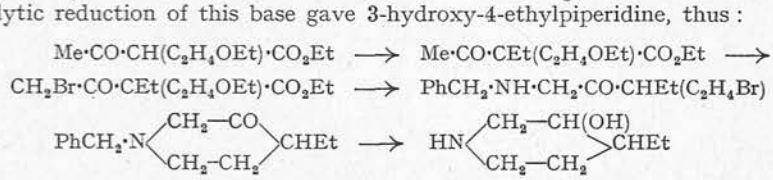


(V.)

As a suitable model for (i), butyraldehyde was successfully condensed with 6-methoxy-4-acetoquinoline using alcoholic potassium hydroxide as a condensing agent to give 6-methoxy-4-quinolyl pentenyl ketone, (III; R = C_3H_7), isolated as the *picrate*.

To extend this synthesis to compounds of the niquidine type phenoxyethylvaleraldehyde was condensed with 6-methoxy-4-acetoquinoline by this method (see following paper).

The practicability of (ii) was examined by synthesising 3-*keto*-N-*benzyl*-4-*ethylpiperidine* by the following series of reactions : *ethyl* α-(β′-*ethoxyethyl*)*acetoacetate* was alkylated with ethyl iodide to give *ethyl* α-*ethyl*-α-(β′-*ethoxyethyl*)*acetoacetate* which was brominated to give *ethyl* γ-*bromo*-α-*ethyl*-α-(β′-*ethoxyethyl*)*acetoacetate*; the latter was condensed with benzylamine and hydrolysed with hydrobromic acid to give *benzyl*-(ε-*bromo*-β-*keto*-γ-*ethylamyl*)*amine hydrobromide*; this on treatment with alkali gave the desired 3-*keto*-N-*benzyl*-4-*ethyl*-*piperidine*. Catalytic reduction of this base gave 3-hydroxy-4-ethylpiperidine, thus :



Condensation of 6-methoxyquinoline-4-aldehyde and 3-keto-*N*-benzyl-4-ethylpiperidine in presence of sodium methoxide yielded α-(3-*keto*-N-*benzyl*-4-*ethyl*)*piperidyl*-6-*methoxy*-4-*quinolylcarbinol* (IV; R = Et, R′ = CH_2Ph), but it was not found possible to reduce the keto to a methylene group (cf. Clemo and Hoggarth, *J.*, 1939, 1241).

As a model for (iii) 6-methoxyquinoline-4-aldehyde was condensed with nitrobutane using sodium methoxide as a condensing agent : 6-*methoxy*-4-*quinolyl*-α-*nitrobutylcarbinol* was obtained in fairly good yield and it was reduced catalytically to 6-methoxy-4-quinolyl-α-aminobutylcarbinol isolated as the *dihydrobromide*. To extend this method to the synthesis of piperidylquinolyl-carbinols, ε-*nitroamyl benzoate*, synthesised from ε-bromoamyl benzoate, was condensed with 6-methoxyquinoline-4-aldehyde and the product catalytically reduced to give 6-*methoxy*-4-*quinolyl*-(α-*amino*-ε-*benzoxyamyl*)*carbinol* (as V; R = $PhCO_2$). This base could not be cyclised to the desired piperidine so the method was modified by using α-*bromo*-ε-*nitropentane* in place of ε-nitroamyl benzoate, bromonitropentane being obtained from αε-dibromopentane by controlled reaction with silver nitrite.

α-Bromo-ε-nitropentane with the bisulphite compound of 6-methoxyquinoline-4-aldehyde and sodium ethoxide (cf. U.S.P. 2,151,517) gave a good yield of 6-*methoxy*-4-*quinolyl*-(ε-*bromo*-α-*nitroamyl*)*carbinol* (V; R = Br). The two externally compensated forms of this compound were separated by fractional crystallisation and reduced catalytically. The yields of the desired α-piperidyl-6-methoxy-4-quinolyl-carbinols were not good owing, apparently, to partial reduction of the ε-bromo group before cyclisation, but both externally compensated forms of the piperidyl carbinol were obtained for comparison with specimens prepared by Ainley and King (*loc. cit.*). One isomeride melted at 166° and was probably identical with that prepared by Ainley and King. The second isomeride melted at 181° and appeared to differ from that reported by Ainley and King.

By the application of this method to a suitably substituted nitropentane, a compound having the structure assigned by Henry and Gibbs (*loc. cit.*) to dihydroniquidine has been synthesised. (See following paper.)

αε-Dinitropentane, available as a by-product in the preparation of bromonitropentane, condensed in the same way with 6-methoxyquinoline-4-aldehyde to give 6-*methoxy*-4-*quinolyl*-αε-*dinitroamylcarbinol* (V; R = NO_2), which was reduced to the corresponding amine isolated as 6-*methoxy*-4-*quinolyl*-αε-*diaminoamyl*-*carbinol trihydrochloride*. In contrast with the corresponding cyclised base, this compound had no antimalarial properties.

EXPERIMENTAL.

Ethyl α-(β'-Ethoxyethyl)acetoacetate.—To ethyl acetoacetate (150 g.) and sodium ethoxide (25 g. Na) in boiling alcohol bromomethyl ether (150 g.) was added slowly. After boiling for twenty-four hours, alcohol was removed and the cooled mixture acidified. The separated product was fractionally distilled under reduced pressure and the fraction (118 g.), b. p. 118—120°/11 mm., was collected (Found: C, 59·4; H, 8·9. $C_{10}H_{18}O_4$ requires C, 59·4; H, 8·9%).

Ethyl α-Ethyl-α-(β'-ethoxyethyl)acetoacetate.—Sodamide (22·6 g.) was added to a solution of the above ethyl ethoxyethylacetoacetate (117 g.) in benzene (300 c.c.) and, when the initial reaction was complete, ethyl iodide (90·6 g.) was added and the solution refluxed for eight hours. The product, isolated in the usual way, was fractionally distilled at reduced pressure and the fraction (91·5 g.), b. p. 132—134°/12 mm., was collected (Found: C, 62·9; H, 9·5. $C_{12}H_{22}O_4$ requires C, 62·6; H, 9·5%).

Ethyl γ-Bromo-α-ethyl-α-(β'-ethoxyethyl)acetoacetate.—To the product from the above preparation (60 g.) in dry chloroform (750 c.c.) was added slowly dry bromine (42 g.) in chloroform (300 c.c.) with stirring and in absence of moisture. The product in chloroform was washed with sodium bicarbonate solution and the solvent removed. The residual oil was distilled and the fraction (60·4 g.), b. p. 142—148°/3 mm., was collected (Found: C, 46·5; H, 7·0. $C_{12}H_{21}O_4Br$ requires C, 46·6; H, 6·8%).

Benzyl-(ε-bromo-β-keto-γ-ethylamyl)amine.—To a solution of ethyl γ-bromo-α-ethyl-α-ethoxyethylacetoacetate (38·4 g.) in dry ether (150 c.c.) a solution of benzylamine (38 g.) in ether (150 c.c.) was added and the mixture left in darkness for 70 hours. Benzylamine hydrobromide (21·1 g.) was collected and the ether solution washed with sodium carbonate. The product was extracted into dilute hydrochloric acid from the ether and liberated by addition of alkali. This oil (35·7 g.) was boiled for four hours with a mixture of hydrobromic acid (45 c.c., *d* 1·7) and glacial acetic acid (150 c.c.). Charcoal was then added to clear the solution, solvent removed under reduced pressure and the residual gum dissolved in 1% hydrobromic acid (800 c.c.), the small insoluble residue being discarded. The product, from the concentration of the solution, crystallised. After crystallisation from aqueous alcohol the *hydrobromide* (26·7 g.) had m. p. 127° (Found: C, 44·4; H, 5·5; Br, 41·6. $C_{14}H_{20}ONBr,HBr$ requires C, 44·3; H, 5·5; Br, 42·2%).

3-Keto-N-benzyl-4-ethylpiperidine.—The crystalline hydrobromide from the previous preparation (20 g.) was suspended in saturated sodium bicarbonate solution and shaken continuously for 24 hours. The *product* was taken up in ether and fractionally distilled under reduced pressure. The fraction (11 g.), b. p. 124°/7 mm., was collected (Found: C, 77·3; H, 8·7; N, 6·3. $C_{14}H_{19}ON$ requires C, 77·4; H, 8·7; N, 6·4%). The base gave a crystalline *picrate*, needles, m. p. 129° (Found: C, 54·1; H, 5·0. $C_{14}H_{19}ON,C_6H_3O_7N_3$ requires C, 53·9; H, 4·9%).

3-Hydroxy-4-ethylpiperidine.—3-Keto-*N*-benzyl-4-ethylpiperidine was reduced at normal pressure in aqueous alcohol with Adams' platinum oxide catalyst. The basic product was isolated as a *picrate*, m. p. 138°, which crystallised as needles from methanol (Found: C, 44·2; H, 5·3; N, 15·2. $C_7H_{15}ON,C_6H_3O_7N_3$ requires C, 43·6; H, 5·0; N, 15·6%).

α-3-Keto-N-benzyl-4-ethylpiperidyl-6-methoxy-4-quinolylcarbinol.—To a solution of 6-methoxyquinoline-4-aldehyde (4·8 g.) and 3-keto-*N*-benzyl-4-ethylpiperidine (5·6 g.) in methanol (10 c.c.) a solution of sodium methoxide (11·3 c.c. containing 0·05 g. sodium per c.c.) was added during one hour. The mixture after 20 hours at 37° was poured into water and the water insoluble fraction taken up in ether. The *base* was taken into acid from ether, liberated by excess alkali and re-extracted with ether. The ether extract (3·5 g.) after removal of solvent crystallised slowly when triturated with benzene and, when pure, had m. p. 127° (Found: C, 74·4; H, 7·0. $C_{25}H_{28}O_3N_2$ requires C, 74·3; H, 6·9%). A crystalline *monopicrate* had m. p. 175° (Found: C, 58·7; H, 5·1; N, 10·9. $C_{25}H_{28}O_3N_2,C_6H_3O_7N_3$ requires C, 58·8; H, 4·9; N, 11·1%).

6-Methoxy-4-quinolyl-α-nitrobutylcarbinol.—α-Nitrobutane (1·03 g.) and 6-methoxyquinoline-4-aldehyde (1·87 g.) were dissolved in alcohol (5 c.c.) and sodium methoxide (4·4 c.c. containing 0·05 g. Na per c.c.) was added very slowly, the mixture warmed to 50° for 5 minutes and then poured into water. The aqueous solution was washed with ether and acidified with dilute acetic acid. The *product* (1·65 g.) after crystallisation from benzene melted at 148° (Found: C, 62·4; H, 6·1; N, 9·7. $C_{15}H_{18}O_4N_2$ requires C, 62·1; H, 6·2; N, 9·6%). An isomer was present in a subsequent experiment but was not obtained in a pure state.

6-Methoxy-4-quinolyl-α-aminobutylcarbinol.—The product from the above preparation was reduced catalytically in ethanol using Adams' platinum oxide. The product crystallised from alcohol in needles as the *dihydrobromide*, m. p. 230° (decomp.) (Found: C, 42·9; H, 5·3; Br, 38·4. $C_{15}H_{20}O_2N_2,2HBr$ requires C, 42·7; H, 5·2; Br, 37·9%).

ε-Nitroamyl Benzoate.—Powdered silver nitrite (14·0 g.) was added slowly with stirring during eight hours to ε-bromoamyl benzoate (20·8 g.) at room temperature; the temperature was then raised to 70—80° and stirring continued for four hours. The silver bromide was separated and the *product* fractionated twice under reduced pressure giving the fraction (10·4 g.) having b. p. 170°/1 mm. (Found: C, 60·5; H, 6·4; N, 6·0. $C_{12}H_{15}O_4N$ requires C, 60·7; H, 6·3; N, 5·9%).

6-Methoxy-4-quinolyl-(α-amino-ε-benzoxyamyl)carbinol.—ε-Nitroamyl benzoate (2·37 g.) was added to solution of sodium ethoxide (0·23 g. sodium) in ethyl alcohol at 0° and, after shaking for thirty seconds, was added rapidly to an aqueous solution of the bisulphite compound of 6-methoxyquinoline-4-aldehyde maintained at a temperature of 50°. The mixture was agitated vigorously for 20 minutes, the precipitated oil extracted with benzene, the benzene washed with dilute bisulphite and then with sodium bicarbonate and the solvent removed. The residual oil was dissolved in methanol and reduced in hydrogen at a pressure of 5 atmospheres in the presence of Raney nickel with carbon dioxide as a buffer (cf. U.S.P. 2,157,391). The product was isolated as a *hydrochloride* (0·9 g.) which on crystallisation from alcohol separated as needles melting with decomposition between 190° and 200° (Found: C, 59·7; H, 6·3; N, 6·2; Cl, 15·3. $C_{23}H_{26}O_4N_2,2HCl$ requires C, 59·1; H, 6·1; N, 6·0; Cl, 15·2%). The *base* crystallised with difficulty from ether and had m. p. 64—66° (Found: C, 70·3; H, 6·6. $C_{23}H_{26}O_4N_2$ requires C, 70·1; H, 6·6%).

α-Bromo-ε-nitropentane.—To αε-dibromopentane (57 g.) in a flask fitted with a high-speed stirrer was added, in small lots, powdered freshly prepared silver nitrite (18 g.) during 3 hours. The reaction was allowed to proceed for a further 48 hours at room temperature and the product fractionally distilled at 15 mm. The first fraction (110—118°) (29·33 g.) was dibromopentane. The intermediate fraction (118—128°) (3·7 g.) was discarded and the final fraction (128—148°) was refractionated and the *distillate* collected between 140—144° (10·0 g.) (Found: N, 7·2. $C_5H_{10}O_2NBr$ requires N, 7·2%).

6-Methoxy-4-quinolyl-(ε-bromo-α-nitroamyl)carbinol.—6-Methoxyquinoline-4-aldehyde (2·2 g.) dissolved in methanol was added to an excess of aqueous sodium bisulphite and the crystalline product collected. This was suspended in water at 40° and to the suspension was added a solution of bromonitropentane (2·4 g.) in alcoholic sodium oxide (2 mols. Na) at 0°. The mixture was shaken vigorously for 30 minutes and the solid collected. Crystallisation from chloroform gave a product, m. p. 130—140°, which was fractionally crystallised from chloroform–ether, two isomeric nitro compounds being isolated; one (1·3 g.) (m. p. 158°) was sparingly and the other (0·65 g.) (m. p. 136—137°) was readily soluble in cold chloroform (Found: C, 50·1; H, 5·0; N, 7·3; Br, 20·7. $C_{16}H_{19}O_4N_2Br$ requires C, 50·1; H, 5·0; N, 7·3; Br, 20·9%).

α-Piperidyl-6-methoxy-4-quinolylcarbinol.—Both isomers were reduced in the same way. The methoxyquinolyl-

bromonitroamylcarbinol was dissolved in methanol, solid CO_2 added, and reduction effected by hydrogen at 5 atms. with Raney nickel catalyst. After removal of the catalyst the product was isolated as a crystalline base. The isomer having m. p. 158° gave a base, m. p. 181°, and the isomer having m. p. 136° gave an isomeric base, m. p. 166°, which, mixed with a specimen of the same base (m. p. 164°) prepared by the method of Ainley and King, gave no depression of the m. p.

6-*Methoxy*-4-*quinolyl*-αε-*dinitroamylcarbinol*.—αε-Dinitropentane was condensed with the bisulphite compound of 6-methoxyquinoline-4-aldehyde by the method described above. The solid product crystallised from alcohol but, although two isomers were obviously present, only one of these, m. p. 163°, was obtained pure; after numerous fractional crystallisations the final *product* separated as sparingly soluble platelets from alcohol (1·1 g.) (Found: C, 55·3; H, 5·4; N, 11·7. $C_{16}H_{19}O_6N_3$ requires C, 55·0; H, 5·4; N, 12·0%).

6-*Methoxy*-4-*quinolyl*-αε-*diaminoamylcarbinol*.—The methoxyquinolyldinitroamylcarbinol was reduced by hydrogen at 5 atms. with Raney nickel as catalyst in the presence of carbon dioxide, an 80% yield being obtained. Methoxyquinolyldiaminoamylcarbinol was isolated as the *trihydrochloride*, m. p. 226° (decomp.), which crystallised as fine needles from methanol–acetone (Found: C, 47·9; H, 6·6; N, 9·6. $C_{16}H_{23}O_2N_3$,3HCl requires C, 48·2; H, 6·5; N, 10·5%).

6-*Methoxy*-4-*quinolyl Pentenyl Ketone*.—6-Methoxy-4-acetylquinoline (1 g.) in alcoholic potassium hydroxide (5 c.c., 5%) was heated to 70° in a flask fitted with reflux condenser, and butyraldehyde (0·36 g.) in alcohol (5 c.c.) was run into the solution. The mixture was boiled for ten minutes, cooled and poured into hydrochloric acid (100 c.c., 1%). The acid was washed with ether and the base liberated by excess alkali. The base (0·7 g.) could not be crystallised but gave a crystalline *picrate*, m. p. 218°, sparingly soluble in cold acetone (Found: C, 54·6; H, 4·3; N, 11·0. $C_{16}H_{17}O_2N,C_6H_3O_7N_3$ requires C, 54·5; H, 4·1; N, 11·6%).

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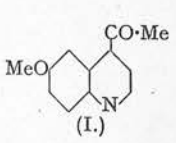
52. *The Synthesis of Antimalarial Compounds Related to Niquidine. Part II. Synthesis of a Dihydro-x-niquidine.*

By T. S. Work.

A synthesis of dihydro-*x*-niquidine has been developed. Condensation of 6-methoxyquinoline-4-aldehyde (IV) with 5-bromo-1-nitro-3-propylpentane (V) gave (VI) which on reduction cyclised spontaneously to give dihydroniquidine (VII). Only one of the four possible racemates was obtained pure. Synthetic dihydroniquidine was found to be a highly active antimalarial.

An alternative synthesis, suggested by preliminary experiments (Part I), the condensation of alkoxyethylvaleraldehydes (II) with 6-methoxy-4-quinolyl methyl ketone (I) was abandoned owing to the impossibility of purifying the product (III).

In Part I (previous paper) general methods for linking the quinoline and piperidine " halves " of the niquidine structure were studied. In order to extend these methods to the synthesis of dihydroniquidine it was necessary to devise methods for the preparation of α(β′-alkoxyethyl)valeraldehyde (II) and *5-bromo-1-nitro-3-propylpentane* (V).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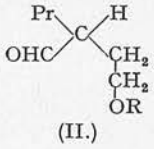
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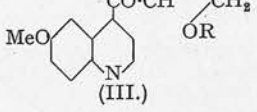
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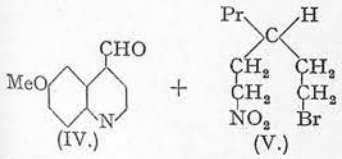
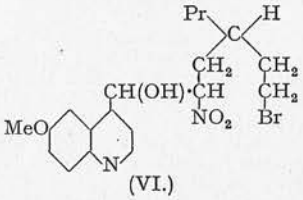
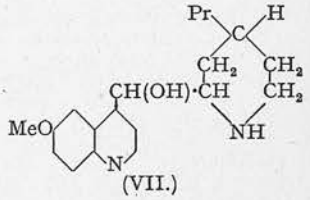
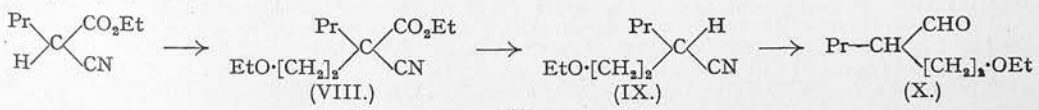

(IV.) (V.)


(VI.)


(VII.)

Ethyl propylcyanoacetate condensed with β-ethoxyethyl bromide to give *ethyl α(β′-ethoxyethyl)propylcyanoacetate* (VIII). Hydrolysis of the ester gave *α(β′-ethoxyethyl)propylcyanoacetic acid* which distilled at low pressure without decarboxylation. Decarboxylation was achieved by distilling slowly at normal pressure to give *α(β′-ethoxyethyl)valeronitrile* (IX). Application of the stannous chloride method of Stephen (*J.*, 1925, 1874) converted this nitrile in rather poor yield into α(β′-ethoxyethyl)valeraldehyde (X) isolated as its 2 : 4-*dinitrophenylhydrazone*.


(VIII.) (IX.) (X.)

fraction, b. p. 104—108°/0·8 mm. (16 g.), was collected (Found : C, 40·3; H, 6·9; N, 6·1. $C_8H_{16}O_2NBr$ requires C, 40·3; H, 6·7; N, 5·9%).

Dihydro-x-niquidine.—To a suspension of the bisulphite compound of 6-methoxyquinoline-4-aldehyde (8·5 g.) in water (100 c.c.) warmed to 40° was added an ice-cold mixture of bromonitro*iso*octane (6·2 g.) and sodium methoxide (1·5 g. Na) in methyl alcohol (20 c.c.). The reactants were shaken vigorously for two minutes and then after another five minutes the supernatant liquid was decanted from the heavy gum which had separated. All efforts to crystallise this product failed. The gum was dissolved in methanol and hydrogenated in the presence of hydrogen and excess of carbon dioxide at room temperature and 5 atmospheres pressure using Raney nickel catalyst. After shaking for four hours the catalyst was removed and the product concentrated. When most of the alcohol had been removed acetone was added and after several hours the crystalline product (1·5 g.) was collected. This material appeared to contain more than one crystalline hydrobromide, but only one compound (0·4 g.) was obtained pure, m. p. 230—231°, after repeated fractional crystallisation from water and from methyl alcohol–acetone. Analytical results showed this to be the *monohydrobromide* of dihydro-*x*-niquidine (Found : C, 58·3; H, 6·9; N, 6·9. $C_{19}H_{26}O_2N_2,HBr$ requires C, 57·7; H, 6·8; N, 7·1%). The base isolated from the hydrobromide was obtained crystalline as a *monohydrate*, m. p. 98—100°, from aqueous acetone (Found : C, 68·5; H, 8·5. $C_{19}H_{26}O_2N_2,H_2O$ requires C, 68·6; H, 8·4%).

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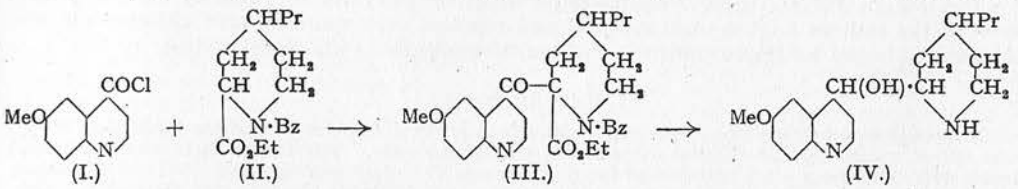
51. *The Synthesis of Antimalarial Compounds related to Niquidine. Part III. Alternative Synthesis of Dihydro-x-niquidine.*

By T. S. Work.

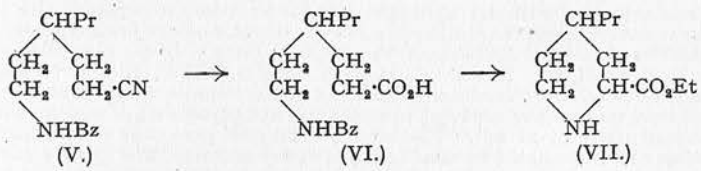
The reaction of quininic acid chloride with ethyl *N*-benzoyl-4-propylpiperidine-2-carboxylate was effected using sodium triphenylmethyl as condensing agent. The resultant keto-ester (III) was decarboxylated and reduced to give a small yield of dihydro-*x*-niquidine.

In the hope of improving upon the synthesis of dihydro-*x*-niquidine already described (Part II, *J.*, 1946, 197) trials were undertaken of the condensation of ethyl quininate with *ethyl* 3-(2-*benzamidoethyl*)*hexane*-1-*carboxylate* by the method of Ainley and King (*Proc. Roy. Soc.*, 1938, *B*, **125**, 60), but results were not encouraging.

As an alternative method the condensation of *ethyl* N-*benzoyl*-4-*propylpiperidine*-2-*carboxylate* (II) with quininic acid chloride (I) was studied (cf. Hudson and Hauser, *J. Amer. Chem. Soc.*, 1941, **65**, 3163). 4-Propylpiperidine was used as starting material for the synthesis of the desired α-carboxylate.



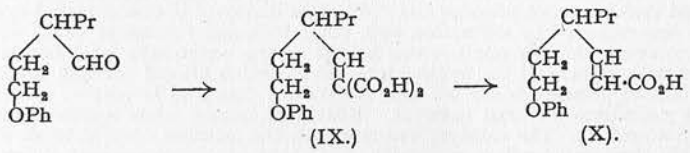
The nitrogen ring of N-*benzoyl*-4-*propylpiperidine* was opened by treatment with phosphorus pentachloride (von Braun, *Ber.*, 1906, **37**, 2916) to give benz-3-(2-chloroethyl)hexylamide. This amide was not purified but was allowed to react with potassium cyanide to yield 3-(2-*benzamidoethyl*)*hexyl cyanide* (V). The cyanide was converted into the *ester* and hydrolysed to the corresponding acid (VI) which was brominated in the α-position to the carboxyl group. Removal of the benzoyl group and cyclisation gave 4-propylpiperidine-2-carboxylic acid, isolated as the ester (VII). Benzoylation of this ester gave (II).



Ethyl *N*-benzoyl-4-propylpiperidine-2-carboxylate (II) with quininic acid chloride (I) and sodium triphenylmethyl in boiling anisole gave the keto-ester (III). This could not be purified, but on hydrolysis with dilute acid carbon dioxide was evolved and benzoic acid liberated. The resultant keto-amine was highly unstable so that it was necessary to handle it only as the hydrochloride and to reduce it as soon as possible to dihydroniquidine (IV). Despite various modifications of the conditions it was not found possible to isolate more than a trace of dihydroniquidine, and in several experiments no crystalline material was isolated.

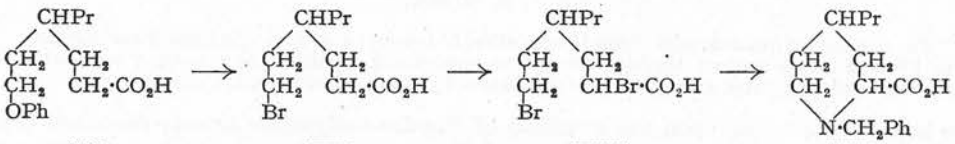
As the small yield appeared to be associated with considerable decomposition during hydrolysis of the benzamido-group, it was thought worth while to replace this by a benzyl group which could be removed by catalytic hydrogenation simultaneously with the reduction of the keto-group to a carbinol.

Phenoxyethylvaleraldehyde (Part II, *loc. cit.*) reacted readily with malonic acid to give (IX) which was decarboxylated to give 3-(2-*phenoxyethyl*)*hex*-1-*en*-1-*carboxylic acid* (X). This acid was



reduced catalytically to 3-(2-*phenoxyethyl*)*hexane*-1-*carboxylic acid* which gave a crystalline S-*benzylthiouronium* salt. Treatment of 3-(2-phenoxyethyl)hexane-1-carboxylic acid with

hydrobromic acid in acetic acid split the ether linkage, and the resultant 3-(2-*bromoethyl*)*hexane*-1-*carboxylic acid* (XII) was brominated in the α-position by treatment with bromine and phosphorus tribromide to give 1-*bromo*-3-(2-*bromoethyl*)*hexane*-1-*carboxylic acid*



(XI. (XII.) (XIII.) (XIV.)

(XIII). The dibromo-acid reacted in the cold with benzylamine, and the resultant acid (XIV) was esterified to give the desired *ethyl* N-*benzyl*-4-*propylpiperidine*-2-*carboxylate*. The *N*-benzyl group was found to be readily removed by catalytic hydrogenation with platinum oxide at 70°.

The use of ethyl *N*-benzyl-4-propylpiperidine-2-carboxylate in place of the *N*-benzoyl ester in the sodium triphenylmethyl-catalysed reaction with quininic acid chloride did not achieve the hoped for improvement in yield in the synthesis of dihydroniquidine.

Experimental.

N-*Benzoyl*-4-*propylpiperidine*.—Propylpiperidine (43 g.) in a solution of sodium hydroxide (21 g.) was stirred vigorously and benzoyl chloride (55 g.) added slowly. The temperature was maintained below 40°. One hour after addition of benzoyl chloride the *compound* was extracted with benzene, dried, and distilled. The fraction (76·2 g.), b. p. 164—168°/0·8 mm., was collected (Found : C, 77·9; H, 9·1; N, 6·4. $C_{15}H_{21}ON$ requires C, 77·9; H, 9·1; N, 6·1%).

3-(2-*Benzamidoethyl*)*hexyl Cyanide*.—*N*-Benzoylpropylpiperidine (94 g.) was mixed with phosphorus pentachloride (78 g.) and heated cautiously under reflux until reaction commenced. When the initial vigorous reaction had subsided the mixture was refluxed for 45 minutes. After cooling, ice was added followed by sodium hydroxide until the solution was just acid to Congo-red paper. The product was steam distilled to remove volatile by-products, and the benz-3-(2-chloroethyl)hexylamide extracted with ether. This oil (82 g.) would not crystallise and was converted into the *cyanide* without purification by heating for 14 hours with potassium cyanide (90 g.) in 75% alcohol (250 c.c.). After removal of solvent the product was extracted with ether and fractionally distilled at 0·7 mm., the fraction (67 g.), b. p. 224—226°, being collected (Found : C, 74·9; H, 8·9. $C_{16}H_{22}ON_2$ requires C, 74·4; H, 8·5%).

Ethyl 3-(2-*Benzamidoethyl*)*hexane*-1-*carboxylate*.—The cyanide from the previous experiment (67 g.) in alcohol (350 c.c.) was saturated with dry hydrogen chloride at room temperature and then boiled for 6 hours. The solvent was removed, the residue diluted with water, and the *product* extracted with ether. The oil was purified by fractional distillation, the fraction (50 g.), b. p. 214—216°/0·7 mm., being collected (Found : C, 70·8; H, 9·0; N, 4·6. $C_{18}H_{27}O_3N$ requires C, 70·8; H, 8·8; N, 4·6%).

Ethyl N-*Benzoyl*-4-*propylpiperidine*-2-*carboxylate*.—3-(2-Benzamidoethyl)hexane-1-carboxylic acid (10 g.) was obtained from the above ester (12 g.) by controlled hydrolysis with potassium hydroxide (2·6 g.) in 60% methyl alcohol (50 c.c.) at 40°. The acid, a colourless gum, was not purified but was dried over phosphoric oxide and brominated by slow addition of dry bromine (22·1 g.) to a stirred mixture of the acid and red phosphorus (1·43 g.). The flask was heated to 100° for 30 minutes to complete the reaction and the product poured on ice. The 1-bromo-3-(2-benzamidoethyl)hexane-1-carboxylic acid obtained in this way was hydrolysed by heating in a sealed tube with concentrated hydrochloric acid (25 c.c.) for 18 hours at 150°. The contents of the tube were diluted with water and benzoic acid extracted with ether. The aqueous solution of 1-bromo-3-(2-aminoethyl)hexane-1-carboxylic acid was concentrated, dried, and esterified with alcohol and dry hydrogen chloride. The acid alcohol was removed under reduced pressure and excess of sodium bicarbonate and ether were added. The solution was shaken for 1 hour to allow time for the cyclisation, and the ether was then separated and dried, and the ether-soluble oil distilled. The ethyl 4-propylpiperidine-2-carboxylate, b. p. 140—144°/18 mm. (3·3 g.), was benzoylated by the method used earlier for benzoyl-4-propylpiperidine, and the benzoate was distilled. The fraction, b. p. 190—194°/1 mm. (3·2 g.), analysed satisfactorily for *ethyl* N-*benzoyl*-4-*propylpiperidine*-2-*carboxylate* (Found : C, 71·3; H, 7·9; N, 4·8. $C_{18}H_{25}O_3N$ requires C, 71·3; H, 8·2; N, 4·6%).

Dihydro-x-*niquidine*.—The above ester (3·2 g.) in dry anisole (5 c.c.) in a dry nitrogen atmosphere was treated with a solution of one equivalent of sodium triphenylmethyl in ether. The colour of the sodium salt disappeared rapidly. Quininic acid chloride (2·3 g.) in anisole (10 c.c.) was added, and ether distilled off. The anisole solution was refluxed in a dry nitrogen atmosphere for 4 hours. The anisole was removed under reduced pressure and the residue dissolved in concentrated hydrochloric acid. Triphenylmethane was removed by extraction with ether, an equal volume of water was added to the hydrochloric acid solution, and the mixture was heated on the water-bath for 4 hours. Benzoic acid was then extracted with ether, and the hydrochloric acid solution filtered through charcoal. The acid was removed at reduced pressure below 60° and the residue dissolved in methyl alcohol and reduced catalytically using palladium–charcoal catalyst. Reduction ceased when approximately 100 c.c. of hydrogen had been adsorbed. The catalyst was removed, the solution concentrated, and the product liberated by addition of aqueous sodium carbonate. The base was extracted with chloroform, and after prolonged manipulation a small quantity of crystalline hydrobromide melting at 224° was isolated. This compound did not depress the m. p. of dihydro-*x*-niquidine (m. p. 229°) prepared by an alternative method (Part II, *loc. cit.*).

3-(2-*Phenoxyethyl*)*hexane*-1-*carboxylic Acid*.—To a solution of malonic acid (9·5 g.) and phenoxyethylvaleraldehyde (18·5 g.) (Part II, *loc. cit.*) in pyridine (40 c.c.), piperidine (0·5 g.) was added as catalyst and after 3 hours the mixture was heated on a boiling water-bath for 2 hours and finally boiled under reflux for 30 minutes. Most of the pyridine was removed under reduced pressure, the residual oil diluted with water, and excess of sodium carbonate added. Unreacted aldehyde (11·0 g.) was recovered by extraction with ether, and the unsaturated acid liberated by addition of hydrochloric acid to the alkaline solution. The acid was decarboxylated and distilled, and the fraction, b. p. 178—180°/0·7 mm., collected. The distillate (8·6 g.) was dissolved in one equivalent of 2N-sodium carbonate and reduced catalytically using palladium–strontium carbonate catalyst. The *product* was purified by distillation, and the fraction (6·3 g.), b. p. 186—188°/1 mm., collected (Found : C, 71·8; H, 8·7. $C_{15}H_{22}O_3$ requires C, 72·0; H, 8·8%). The S-*benzylthiouronium* salt of the acid crystallised from acetone melted at 117° (Found: C, 66·9; H, 7·4; N, 6·4. $C_{15}H_{21}O_3,C_8H_{11}N_2S$ requires C, 66·4; H, 7·7; N, 6·7%). The *ethyl* ester boiled at 204°/12 mm. (Found : C, 73·8; H, 9·2. $C_{17}H_{26}O_3$ requires C, 73·4; H, 9·3%).

3-(2-*Bromoethyl*)*hexane*-1-*carboxylic Acid*.—3-(2-Phenoxyethyl)hexane-1-carboxylic acid (50 g.) in a mixture of hydrobromic acid (200 c.c.; *d* 1·7) and glacial acetic acid (560 c.c.) was boiled under reflux for 7 hours. The solvent was removed under reduced pressure and the residue diluted with water. The *bromo-acid* was extracted with ether, extracted from the ether with sodium carbonate solution, and liberated by addition of excess of hydrochloric acid. It was purified by fractional distillation, the fraction (32 g.), b. p. 164—168°/3 mm., being collected (Found : Br, 33·1. $C_9H_{17}O_2Br$ requires Br, 33·7%).

1-*Bromo*-3-(2-*bromoethyl*)*hexane*-1-*carboxylic Acid*.—The above acid (32 g.) and phosphorus tribromide (0·5 c.c.) were mixed, and dry bromine (31·5 g.) was added slowly to the stirred solution under anhydrous conditions. When addition was complete the mixture was heated and maintained at 75° for 15 hours. The temperature was finally raised to 110° for 10 minutes and the product distilled, the fraction, b. p. 180—190°/3 mm., being collected. Redistillation gave the required *acid* (46 g.), b. p. 144—146°/0·6 mm. (Found : C, 34·4; H, 5·2; Br, 49·5. $C_9H_{16}O_2Br_2$ requires C, 34·2; H, 5·1; Br, 50·6%).

Ethyl N-*Benzyl*-4-*propylpiperidine*-2-*carboxylate*.—The bromo-acid from the above experiment (40 g.) was mixed with methyl alcohol (40 c.c.), cooled in ice, and benzylamine (40 g.) in methyl alcohol (40 c.c.) added slowly. After 2 hours at 0° and 24 hours at room temperature the mixture was heated for 3 hours at 90°. Methyl alcohol was removed and the product shaken with ether and excess of dilute sodium hydroxide. The sodium *N*-benzylpropylpiperidine-2-carboxylate was sparingly soluble in sodium hydroxide, but dissolved on adding sufficient water. The ether was separated and discarded. On addition of excess of 50% sodium hydroxide to the aqueous solution the sodium salt separated and was extracted with butyl alcohol. The butyl alcohol extract was concentrated and the residue esterified by treatment with alcohol and dry hydrogen chloride. The *ethyl* N-*benzyl*-4-*propylpiperidine*-2-*carboxylate* was fractionally distilled twice, and the fraction (9·4 g.), b. p. 174—176°/1 mm. collected (Found : C, 75·2; H, 9·2; N, 5·1. $C_{18}H_{27}O_2N$ requires C, 74·7; H, 9·3; N, 4·8%).

Ethyl 4-*Propylpiperidine*-2-*carboxylate*.—The distillate from the above preparation (6·0 g.) in glacial acetic acid (50 c.c.) was reduced catalytically at 70° and normal pressure with Adams's platinum oxide catalyst. Reduction was complete in 6 hours. The catalyst was removed, the solvent distilled at reduced pressure, and the base liberated by addition of saturated sodium carbonate solution. The *base* after fractional distillation had b. p. 140—142°/18 mm. (2·5 g.) (Found : C, 66·4; H, 10·4; N, 7·2. $C_{11}H_{21}O_2N$ requires C, 66·3; H, 10·5; N, 7·0%).

I wish to thank Mr. N. Schunman for valuable technical assistance. Dr. E. R. Buchman (private communication) has successfully applied the sodium triphenylmethyl method in similar condensations of ethyl *N*-benzoylpiperidine-2-carboxylate with aromatic esters and reports equally disappointing yields. I am greatly indebted to Mr. Solomon of the Wellcome Research Institution, who developed the same method independently, for advice on the experimental technique.

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A New Antimalarial Drug

THROUGH the courtesy of Dr. J. Needham of the British Scientific Office in Chungking, samples of two indigenous plants regarded locally as of value in the treatment of malaria have been obtained and tested in experimental animals.

One of these, *Fraxinus malacophylla*, was found to be without action on *Plasmodium gallinaceum* in chicks. Contrary to the report of Liu *et al.*[1], no alkaloid could be found in the bark.

The root of a second plant, known locally as 'chang shan' and reputed to be *Dichroa febrifuga*, Lour., was ground and extracted with water and with dilute acid. Neither extract at a concentration where 1 c.c. corresponded to 1 gm. dry root gave the usual alkaloidal reactions, but both showed considerable activity against a trophozoite-induced infection of *P. gallinaceum* in chicks. The growth of exo-erythrocitic forms was not prevented by the extract.

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[1] Liu, Chang, Ch'uan and Tan, *Chinese Med. J.*, **59**, 573 (1941).

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7. D- AND L-AMINO-ACIDS IN ANTIBIOTICS

By T. S. WORK
National Institute for Medical Research, London

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7. D- AND L-AMINO-ACIDS IN ANTIBIOTICS

BY T. S. WORK
National Institute for Medical Research, London

The term 'antibiotic' owes its origin to Waksman, and is derived from the much older word 'antibiosis', that is, interference with growth of one micro-organism by another. An antibiotic may be defined as a chemical substance produced by any micro-organism which has the power to inhibit growth in another micro-organism. Used in this wide sense, antibiotic action has been recognized for more than 50 years (Waksman, 1945).

Pasteur & Joubert (1877) observed an antagonistic relationship between anthrax and certain aerobic bacteria. Later, Babès (1885) suggested that inhibition of growth was caused by a definite chemical substance produced during bacterial growth and, in the period from 1885 to 1939, numerous attempts were made at extraction of antibiotic principles which might have therapeutic value. Pyocyanase, the preparation of Emmerich & Low (1899), was used with some success in local infections, but gradually fell into disrepute. Perhaps the original active strain of *Pseudomonas pyocyanea* was contaminated or lost during commercial preparation of pyocyanase, since recent investigation has indicated that antibiotics are produced by this organism (Hays, Wells, Katzman, Cain, Jacobs, Thayer, Doisy, Gaby, Roberts, Muir, Carroll, Jones & Wade, 1945).

In 1907 Nicolle demonstrated the production of a bactericidal substance by *Bacillus subtilis*, but it was not until the preparation in 1939 of tyrothricin from the related organism *B. brevis* that the isolation of naturally occurring antibiotics began to be regarded as a practical proposition (Dubos, 1939; Dubos & Cattaneo, 1939). The demonstration in the following year of the therapeutic possibilities of penicillin (Chain, Florey, Gardner, Heatley, Jennings, Orr-Ewing & Sanders, 1940) stimulated extensive research, so that to-day new antibiotics appear with bewildering frequency. Unfortunately, the majority of these are toxic to animals, as well as to micro-organisms, so that their fame is as short-lived as last year's 'best-seller'.

From much recent work the conclusion can be drawn that aerobic sporulating bacteria, as a group, tend to be antagonistic towards other micro-organisms, and frequently produce soluble antibiotics which are sufficiently stable to permit chemical isolation. Tyrothricin, extracted from an autolysate of *B. brevis*, has been resolved into two peptides,

gramicidin and tyrocidine (Hotchkiss & Dubos, 1940, 1941; Tishler, Stokes, Trenner & Conn, 1941). Gramicidin-S, also a peptide, has been isolated by Russian workers from aerobic sporulating bacilli, and another antibiotic peptide, subtilin, has been extracted from *B. subtilis* (Gause, Brazhnikova, Belozersky & Passhina, 1944; Salle & Jann, 1946). In addition, low molecular weight antibiotics of unknown chemical nature have been obtained from other organisms of the same group, licheniformin from *B. licheniformis* (Callow & D'Arcy Hart, 1946), bacillin, bacitracin and subtilysine from *B. subtilis* (Foster & Woodruff, 1946; Johnson, Anker & Meleney, 1945; Vallee, 1945), another subtilin from another strain of *B. subtilis* (Jansen & Hirschmann, 1944), colistatin from an unidentified aerobic bacillus (Gause, 1946) and still other antibiotics from other related bacilli (McDonald, 1940; Stokes & Woodward, 1942; Olivier, 1946).

Because of the difficulties involved in analyzing macromolecules, an endless controversy on the structure of proteins has littered the pages of scientific journals since the pioneer investigations of Emil Fischer, and it is somewhat surprising that more determined efforts were not made before 1939 towards the isolation of naturally occurring polypeptides which might be amenable to chemical investigation. Prior to the isolation of gramicidin, only two natural peptides had been studied in any detail, salmine and clupein, and these were probably mixtures of whole series of closely related compounds, rather than definite chemical entities (Felix & Mager, 1937).

During the last six years, considerable progress has been made in the analysis of the structure of antibiotic peptides; the advances up to 1943 have been summarized by Hotchkiss (1944).

The gramicidin of Dubos, which will be referred to subsequently as gramicidin-D to avoid any possibility of confusion, exists along with tyrocidine in the crude culture autolysate from *B. brevis* in a water-soluble form, and may conceivably be only part of a larger protein molecule; more probably, however, it exists in loose physical combination with a soluble macromolecule which absorbs and holds in solution the water-insoluble peptide. Acidification of the autolysate gives a precipitate from which the crude antibiotic peptides can be isolated, either in a water-soluble form by extraction with neutral buffer, or in a water-insoluble form by extraction with alcohol (Hotchkiss & Dubos, 1941). By making use of the ability of tyrocidine to form an ether-insoluble hydrochloride, gramicidin, which does not form a hydrochloride, can be isolated by continuous extraction with ether and the remaining tyrocidine hydrochloride crystallized from alcohol (Hotchkiss & Dubos, 1941; Tishler *et al.* 1941). Although gramicidin-D and tyrocidine are the only two crystalline antibiotics isolated from *B. brevis*

cultures, Gordon, Martin & Synge (1943*a*) have suggested that other closely related peptides may remain in the mother liquors from the crystalline material.

Following upon the recognition of the peptide nature of gramicidin-D and tyrocidine, a considerable number of papers has been published on the amino-acid composition of the two antibiotics. Tryptophan, leucine, valine, alanine and glycine have been identified as products of acid hydrolysis of gramicidin-D (Hotchkiss & Dubos, 1941; Lipmann, Hotchkiss & Dubos, 1941; Hotchkiss, 1941; Christensen, Edwards & Piersma, 1941; Gordon *et al.* 1943*a*). In addition to these amino-acids the only other product of acid hydrolysis identified was ethanolamine (Synge, 1945*a*). Tryptophan and alanine probably occur entirely in the 'natural' (L) form, but the leucine isolated after acid hydrolysis of gramicidin-D had the 'unnatural' (D) configuration, and valine appeared as DL-valine, a result that can only be explained by the assumption that D- and L-valine exist at different points in the original peptide (unless, of course, valine is assumed to be racemized during isolation). After incomplete acid hydrolysis, Christensen isolated D-valyl-D-valine and L-valyl-L-valine (Christensen, 1943, 1944; Christensen & Hegsted, 1945), but Synge (1944) obtained L-valylglycine. Synge's analytical figures indicated that two L-valine residues per gramicidin molecule were linked through their carboxyl groups to glycine, and that only three other valine residues were available to account for the formation of both D-valyl-D-valine and L-valyl-L-valine. Synge has suggested that steric factors might necessitate inversion of both asymmetric centres simultaneously, and thus explain the production of equimolar quantities of DD- and LL-valylvaline, but no example of such a change has been reported. At present it is difficult to reconcile these results except by the assumption that gramicidin-D is itself a mixture of two optically isomeric peptides.* There seems no doubt, however, that gramicidin-D does contain two amino-acids, leucine and valine, in the D-form. All investigators are agreed that gramicidin-D contains no free amino- or carboxyl groups, but there is good evidence for the existence of two free hydroxyl groups per molecule. Molecular-weight estimations by physical methods vary considerably, but the values most consistent with analytical data are of the order of 3000 (Tishler *et al.* 1941; Hotchkiss, 1944). If the molecule is of this size, analytical data can be best inter-

* Synge (private communication) states that recent quantitative analyses of hydrolysates of gramicidin-D establish that the hydrolysis procedures used in previous analyses were insufficient to liberate all the valine from peptide linkage. In the light of the new data it is simplest to postulate a stoichiometric minimum unit, having 20 N atoms (wt. approx. 2,000) made up from 4 residues each of leucine, tryptophan and valine (2 D and 2 L), 2 of alanine, and 1 each of glycine and ethanolamine. This postulate accounts completely for the C of gramicidin-D and offers a simple explanation of the origin of the peptides of valine so far isolated from partial hydrolysates.

preted as indicating the amino-acid composition set out in Table 1 (Synge, 1945*a*).

If the ethanolamine residues are involved in peptide linkage through their amino-groups and have their hydroxyl groups free, then the two reactive OH groups of gramicidin are satisfactorily accounted for, but it can be found by trial that no way exists by which the postulated amino-acid residues can be joined in peptide linkage along with two ethanolamine residues without leaving some unsubstituted (i.e. reactive) basic groups. In other words, some small residue may have been overlooked; not an impossible assumption, since much of the analytical work has been carried out with lots of a few milligrams of a compound of mol.wt. 3000!

Table 1. *Gramicidin-D*

Component	No. of residues per molecule (molecular weight *c.* 3000)	Reactivity of molecule
L-Tryptophan	6	—
D-Leucine	6	No NH_2
L- and D-valine	5	No COOH
L-Alanine	3	—
Glycine	2	—
Ethanolamine	2	2OH

From the above brief outline of the chemistry of gramicidin-D it is obvious that despite great technical advances introduced by Gordon *et al.*, it is impossible to do more than assert the peptide nature of gramicidin-D, and affirm the presence in the molecule of five amino-acids, one entirely in the D-form and another partly of 'unnatural' configuration.

Tyrocidine, the second component of crude tyrothricin, has been submitted to similar methods of chemical analysis and nine amino-acids identified as products of acid hydrolysis (Hotchkiss, 1941; Christensen *et al.* 1941; Gordon *et al.* 1943*b*; Christensen, Uzman & Hegsted, 1945). Tyrocidine, unlike gramicidin-D, possesses an ionizable basic group and forms a stable hydrochloride, so that it is possible to estimate its equivalent weight with fair accuracy as 1285 (± 50) (Hotchkiss, 1941), and the empirical formula as $C_{63}H_{83}N_{13}O_{13}$, HCl. Hotchkiss (1941, 1944) suggests a molecule of double this size as most consistent with analytical figures. Mild acid hydrolysis of tyrocidin liberates 3 mol. of labile ammonia per molecular unit of this size.

Comparison of the analytical results of Hotchkiss (1941), Gordon *et al.* (1943*b*) and Christensen *et al.* (1945), suggests that the molecule is built up from the amino-acids listed below (Table 2), and that the labile NH_3 is satisfactorily accounted for as ω-amido-groups of the dicarboxylic acids. By reaction of tyrocidine with *S*-methyl*iso*thiourea followed by acid hydrolysis Christensen (1945) was able to show that the δ-amino-groups of ornithine were unsubstituted in the original molecule. Ornithine was converted quantitatively into L-arginine by this procedure, and no other

alkylguanidinium groups were formed. Reaction of tyrocidine with *p*-toluenesulphonyl chloride followed by hydrolysis led to isolation of *O*-*p*-toluenesulphonyl-L-tyrosine and δ-*p*-toluenesulphonyl ornithine. The free hydroxyl groups of the two tyrosine residues, the free δ-amino groups of the two ornithine residues and three ω-amido groups of aspartic or glutamic acid, thus account completely for the reactivity of tyrocidine. If it is assumed that tyrocidine is built up from the nineteen amino-acids listed in Table 2, linked together in a peptide chain, then in order to account for the absence of free α-NH_2 groups, it is also necessary to assume that the molecule is a cyclopeptide.

Table 2. *Tyrocidine*

Amino-acids	No. of residues per molecule (molecular weight *c.* 2500)	Reactivity of molecule
L-Ornithine	2	2 reactive NH_2
L-Proline	2	—
L-Leucine	2	—
L-Valine	2	—
L-Tryptophan	2(?)	—
L-Tyrosine	2	2 reactive OH
L-Aspartic acid	2	3 acid labile NH_2
L-Glutamic acid	2	
Labile NH_2	3	
D-Phenylalanine	3	—

It is apparent that if the single somewhat novel suggestion of a cyclic structure is made, the analytical and chemical results for tyrocidine present a more satisfactory and complete picture than has been obtained for gramicidin-D.

Although gramicidin-D and tyrocidine are produced simultaneously by the same organism they only have two amino-acids in common, namely, L-tryptophan and L-valine. The bacillus produces at the same time one peptide containing D-leucine and another containing L-leucine, and, moreover, it incorporates in a single molecule tyrosine of L-configuration and its assumed precursor phenylalanine in D-configuration. These facts indicate that in *B. brevis* some amino-acid synthesis may be asymmetric, a conclusion in contrast to that reached for animals where amino-acid synthesis is said to be symmetric (Shemin & Rittenberg, 1943).

Another antibiotic gramicidin-S resembles tyrocidine in several respects. Acid hydrolysis indicates that the five amino-acids, valine, leucine, proline, ornithine and phenylalanine, are joined in peptide linkage. Phenylalanine has the 'unnatural' (D) configuration as in tyrocidine; the other four amino-acids are probably in the L-form. Reaction of gramicidin-S with dinitrofluorobenzene followed by acid hydrolysis gave δ-dinitrophenyl-L-ornithine as the only dinitrophenyl amino-acid (Sanger, 1946), so that, as in tyrocidine, the δ-amino-group

of ornithine is apparently the only reactive amino-group. Quantitative analysis of gramicidin-S by Synge (1945*b*) has indicated that the five amino-acids are present in equimolar proportions, but Belozersky & Passhina (1945) have published very different analytical figures, so that for the present no agreed figures for the proportions of different amino-acids can be quoted. Fractionation of partial hydrolysates by the new technique of two-dimensional partition chromatography (Consden, Gordon & Martin, 1944) has led to the recognition of four dipeptides and three tripeptides (Table 3) as products of partial hydrolysis (Consden, Gordon, Martin & Synge, 1946). X-ray crystallographic data of Crowfoot and Schmidt (unpubl.) indicate a pentapeptide molecule, or a decapeptide molecule with crystallographic twofold symmetry. Consden *et al.* (1946) suggest that the molecule may be a cyclic decapeptide. Molecular weight determinations are in agreement with this assumption (Belozersky and Passhina, 1945). The arrangement of amino-acids in the peptides identified by Consden *et al.* (1946) suggests that the five amino-acid residues in gramicidin-S occur as the sequence 'valylornithyl-leucyl-phenylalanylprolyl'.

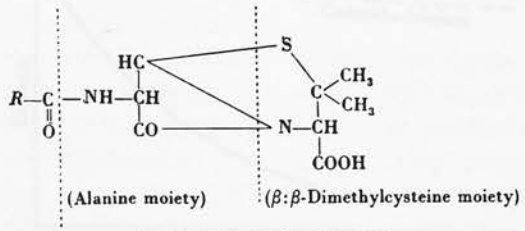
Table 3. *Gramicidin-S*

Amino-acids	Reactivity of molecule	Peptides formed during partial hydrolysis
L-Valine	—	Valyl-ornithine
L-Leucine	—	Ornithyl-leucine
L-Proline	—	Leucyl-phenylalanine
L-Ornithine	Reactive NH_2	Phenylalanyl-proline
D-Phenylalanine	—	Valyl-ornithyl-leucine, phenylalanyl-prolyl-valine, prolyl-valyl-ornithine

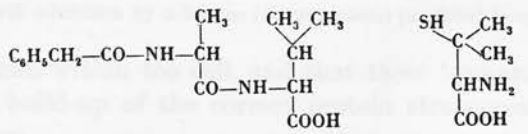
Although the assumption of a cyclic structure for these peptides is somewhat novel to protein chemistry, other, unconfirmed, reports of the occurrence of cyclic peptides in nature have appeared. The toxin phalloidine, $C_{30}H_{39}O_9N_7S$, from the fungus *Amanita phalloides*, contains cysteine, alanine, hydroxyproline and hydroxytryptophan. No free NH_2 or COOH groups are present. The absence of these groups would indicate a cyclopeptide structure (Wieland & Witkop, 1940). The closely related toxin amanitine, also a peptide, has not been studied in detail (Wieland, Hallermeyer & Zilg, 1941). Another cyclopeptide, $C_{16}H_{29}O_3N_3$, has been reported as a product of brief acid hydrolysis of the mollusc *Pecten islandicus* (Sadikov & Kristalinskaya, 1937), and a cyclopeptide, $C_{23}H_{42}N_4O_4$, believed to be *cyclo*leucyl-isoleucyl-*iso*valyl-isoleucine, has been obtained by heating yeast with sodium carbonate (Sadikova, 1939). It is by no means clear whether these last two compounds occur as such or whether they are artefacts produced during chemical manipulation. It is noteworthy that the cyclic peptides which have been examined in detail, gramicidin-S, tyrocidine and phalloidine, all contain amino-

acids of unnatural configuration. It may be that cyclopeptide formation demands that all amino-acids be not of the same configuration. Crowfoot (private communication) has expressed the opinion, from a study of models, that D-configuration of some of the constituent amino-acids in a decapeptide would facilitate cyclization.

No detailed account of the chemistry of the most important of all antibiotics, penicillin, is yet available, but sufficient has been disclosed in general reports (O.S.R.D. and M.R.C. 1945; du Vigneaud, Carpenter, Holley, Livermore & Rachele, 1946) to make it clear that penicillin is closely related to the amino-acids. Hydrolysis with dilute mineral acid gives rise to the amino-acid penicillamine (III) (β:β-dimethylcysteine), and reduction with Raney nickel gives (with penicillin II) phenylacetyl-L-alanyl-D-valine (II). These changes are indicated in the formulae (I)–(III) below where (I) represents the probable formula of penicillin.



(I) Penicillin II ($R = C_6H_5CH_2$)

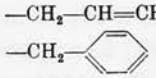


(II) Phenylacetyl-L-alanyl-D-valine

(III) β:β-Dimethylcysteine (penicillamine)

The biologically important part of the molecule apparently resides in the structure built up from the two amino-acids L-alanine and D-valine, since the benzyl group of penicillin II can be replaced by various other groups without loss of antibiotic action. Several naturally occurring antibiotic penicillins are listed in Table 4 below.

Table 4. *Natural penicillins*

Trivial names	Structures R (in formula (I))
Penicillin I or F	$-CH_2-CH=CH-CH_2-CH_3$
Penicillin II or G	
Penicillin III or X	
Penicillin IV or K	$-CH_2-(CH_2)_5-CH_3$

The repeated demonstration of D-configuration among amino-acid constituents of antibiotics naturally suggests that the optical configuration of these amino-acids has some special significance in relation to antibiotic action, and this view is supported, at first sight, by the demonstration by the school of du Vigneaud (1946) that antibiotic penicillin may be synthesized from D-cysteine or D-penicillamine, whereas the isomer from L-penicillamine is biologically inactive.

According to Hotchkiss (1944) and Gale & Taylor (1946*a*) the antibiotics gramicidin-D and penicillin prevent cell division by interference with cell synthesis rather than by inhibition of energy-yielding oxidations. As a working hypothesis which might explain the inhibition of synthesis and the specific effect of peptides containing D-amino-acids it could be supposed that antibiotic D-amino-acid peptides liberated

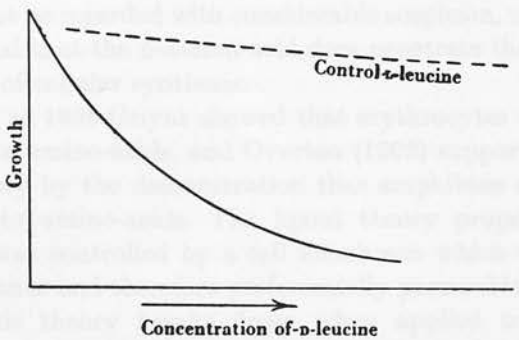


Fig. 1. Growth inhibition by D-leucine (*L. arabinosus*) (modified from Fling & Fox, 1945).

D-amino-acids within the cell, and that these 'unnatural' amino-acids prevented build-up of the correct protein structures essential to cell reproduction.

Such a hypothesis implies that D-amino-acids in nutrient medium would be expected to show antibiotic action. The majority of investigators are agreed that such micro-organisms as *Neurospora* mutants which are unable to synthesize certain amino-acids are able to utilize D-amino-acids as growth factors because they possess enzyme systems which convert added D-amino-acid to the corresponding L-form (cf. Horowitz, 1944). In cultural conditions where growth response is proportional to the amount of L-amino-acid added to the medium, the organisms *Lactobacillus arabinosus*, *L. casei* and *Streptococcus faecalis* give exactly half the normal growth response when a DL-mixture is used in place of pure L-isomer; in other words, the D-isomer of an amino-acid behaves as a physiologically inert compound (Stokes, Gunness, Dwyer & Caswell, 1945; Stokes & Gunness, 1944). One exception has been reported, *Lactobacillus arabinosus* appears to be able to utilize D-glutamic

acid to a very limited extent (Dunn, Camien, Rockland, Shankman & Goldberg, 1944; Lewis & Olcott, 1945). By increasing the ratio of D-amino-acid to L-amino-acid in the nutrient medium, Fling & Fox (1945) were able to show that the D-isomers of leucine and valine are not in fact physiologically inert for *L. arabinosus*, but at suitable concentrations exerted quite considerable growth inhibitory action (Fig. 1). Inhibition only became significant when the ratio of D/L was greater than 40/1. Obviously minimal growth-inhibitory concentrations of antibiotics such as gramicidin-D or penicillin could not give sufficient D-amino-acid to raise the ratio of D-acid/L-acid in normal media to anything approaching 40/1, but, nevertheless, an extracellular ratio D/L of 40/1 does not necessarily imply that this ratio would extend within the cell to the point of cell synthesis. The absence of growth inhibition by a D-isomer must be regarded with considerable suspicion, unless evidence can be provided that the D-amino-acid does penetrate the cell wall and reach the site of cellular synthesis.

As long ago as 1896 Griyns showed that erythrocytes were virtually impermeable to amino-acids, and Overton (1902) supported his lipoid-solubility theory by the demonstration that amphibian cells were also impermeable to amino-acids. The lipoid theory proposed that cell permeability was controlled by a cell membrane which was composed of fatty substance and therefore preferentially permeable to fat soluble molecules. This theory breaks down when applied to the sulphur bacterium *Baggiatoa mirabilis*, where the cell membrane appears to behave as a non-lipoid molecular sieve, but, nevertheless, *Baggiatoa* is found to be relatively impermeable to amino-acids (Ruhland & Hoffmann, 1925; Schonfelder, 1930; Davson & Danielli, 1943). The relative impermeability of inert collodion membranes to amino-acids was also demonstrated (Schmengler, 1933). Since there is no doubt that amino-acids do pass readily through some cell membranes, Hober & Hober (1937) were led to suggest that amino-acids were subject to a mechanism of active transport across the cell wall. Knaysi (1946) has produced convincing evidence that bacteria, like other cells, possess a plasma membrane which may be expected to show differential permeability.

Broadly speaking, micro-organisms fall into two groups. One group, including many Gram-negative organisms, has relatively simple nutritional requirements and does not require to draw amino-acids from the nutrient medium; the second group, including many Gram-positive organisms, requires highly complex media from which essential amino-acids are supplied during growth (Gale, 1947). This second group has relatively limited synthetic powers and lacks many enzymes present in the first group. Taylor (1947) has found that eleven Gram-negative organisms were impermeable to certain amino-acids, but that sixteen

Gram-positive organisms were able to assimilate these amino-acids. This does not mean that the cell membranes were necessarily permeable to amino-acids, but rather that an active transport mechanism exists; thus Gale (1947) found that resting cells of *Streptococcus faecalis* were impermeable to glutamic acid, glutamine or histidine, but that in the presence of a suitable energy source this organism possessed a mechanism for the active transport of those amino-acids across the cell membrane. An active transfer mechanism may be expected to be optically specific so that the *d*-isomers of amino-acids may never reach the interior of *S. faecalis*. Whatever the metabolic apparatus for the transformation of D-glutamic to L-glutamic acid within the cell it would thus have no opportunity to function, and whatever the potential inhibitory action of D-glutamic acid within the cell it could not be measured by exposure of the organism to a medium containing D-glutamic acid. It is possible, of course, that an optically specific transport mechanism might itself be inhibited by 'unnatural' amino-acids, in which case a competitive antagonistic relationship would be expected to exist between a D-amino-acid and its L-isomer. Evidence that failure to metabolize a D-amino-acid cannot always be attributed to lack of suitable enzymes within the cell may be deduced from the results of Bernheim, Bernheim & Webster (1935) and of Stumpf & Green (1944, 1946), which are summarized in the accompanying Table 5.

Table 5. *Comparative metabolic capacity of resting cells and cell-free extracts* (Bacillus proteus)

	Metabolism by washed suspension		Amino-acid oxidase activity of cell-free extract	
Amino-acid	L	D	L	D
Serine	+	?±	.	.
Methionine	+	−	+	+
Alanine	+	?±	.	+
Phenylalanine	+	−	+	+
Tyrosine	+	−	+	.
Tryptophan	+	−	+	.
Proline	+	−	.	.
Leucine	+	−	+	.
Glycine	+	−	.	.
Histidine	.	.	+	+

It is apparent that the failure of a resting suspension of *Bacillus proteus* to metabolize D-methionine and D-phenylalanine cannot be attributed to lack of suitable enzymes but rather to the fact that these amino-acids were unable to penetrate the cell wall of this Gram-negative organism.

Bearing in mind the complexities introduced by permeability effects the general failure of D-amino-acids to show a growth-inhibitory effect comparable to the D-amino-acid antibiotics does not eliminate the

possibility that antibiotic action is related to the occurrence of 'unnatural' amino-acids in these molecules. From the point of view of mechanism of antibiotic action knowledge of the ratio of D-amino-acid to L-isomer at the point of cell synthesis would be more significant than the ratio D/L in the nutrient medium.

The difficulty of arriving at any convincing answer to such a problem is particularly clearly demonstrated by the study of inhibition by arginine of the *Neurospora* mutant 'lysine-less'. *Neurospora* contains an active D-amino-acid oxidase capable of oxidizing sixteen amino-acids, including methionine, leucine, arginine and lysine (Horowitz, 1944). The mutant strains 'methionine-less', 'leucine-less' and 'arginine-less' can oxidize and utilize DL-amino-acids completely, in other words they can convert D-methionine, D-leucine and D-arginine through the keto-acids to the corresponding L-isomers. As might be expected, they are able to grow also on the keto-acids. The 'lysine-less' strain cannot grow on D-lysine, although *Neurospora* amino-acid oxidase can oxidize D-lysine, therefore the 'lysine-less' strain must be unable to convert keto-ω-aminocaproic acid to L-lysine (in passing it may be remarked that animals are also unable to aminate this keto-acid). Growth of the 'lysine-less' mutant on L-lysine can be inhibited by L-arginine but not by D-arginine as indicated in Table 6. Nevertheless, the 'arginine-less'

Table 6. *Inhibition of 'lysine-less'* Neurospora *by arginine* (data from Doermann (1944))

Amino-acid mixture	% growth response
L-Lysine	100
D-Lysine	0
L-Lysine + D-arginine	100
L-Lysine + L-arginine	50

mutant, and therefore presumably the 'lysine-less' mutant can convert D-arginine to L-arginine. The only interpretation which can be put on these results is that conversion of D-arginine to L-arginine does not take place at the same point in the cell as utilization of L-lysine, in other words cell organization extends inside the plasma membrane, and interpretation of results obtained by exposure of intact organisms to 'unnatural' enantiomorphs of amino-acids must take this factor also into account. This view is confirmed by the complete failure of L-arginine to inhibit growth of the 'wild-type' *Neurospora* which can synthesize its own requirements of L-lysine. That arginine inhibition was not due to a second gene mutation separate from the one concerned with lysine synthesis was indicated by examination of thirty separately occurring 'lysine-less' strains known to be of at least three genetic types, all of which showed the same phenomenon (Doermann, 1944).

Numerous other examples implying internal organization of enzymes in micro-organisms can be culled from the field of drug antagonism.

The pitfalls and uncertainties introduced by the complications of cell permeability and cell organization render argument derived from experiments with intact organisms and D-amino-acids somewhat hazardous, so that, despite the often-repeated objection that separated enzyme systems cannot be regarded as a guide to the behaviour of the normally metabolizing organism, one is forced to consider whether any evidence can be deduced from experiments in isolated enzyme systems which would indicate that D-amino-acids might be expected to inhibit cell synthesis.

The evidence that antibiotics prevent cell synthesis would suggest examination of the effect of D-amino-acids on enzymes which utilize the corresponding L-isomers in synthetic reactions. Experiments using labelled nitrogen have demonstrated, at least in animals, that L-amino-acids supplied in the diet are built up into proteins without preliminary deamination. The suggestion has been put forward several times that peptide bond formation may occur not by condensation of amino-acid with amino-acid but by condensation of keto-acid with amino-acid followed by amination of the resultant ketoamide (Lindstrøm-Lang, 1939). There is no convincing evidence for such a mechanism, and it is more probable that peptide formation is catalyzed by the peptidases. Peptidases tend to be regarded as hydrolytic enzymes incapable of catalyzing peptide bond formation (an endergonic process), but Behrens & Bergmann (1939) showed that 'amide interchange', which is not an endergonic process, was catalyzed by these enzymes. Systems exist for the biological acetylation of amino-acids (Bloch & Borek, 1946), and it is quite likely that acetyl phosphate plays a part in such processes. Replacement of an acetyl group by an amino-acid residue is an 'amide interchange' which does not require a source of energy and could proceed in the same way as peptidase-catalyzed amide interchange.

Full information on the effect of D-amino-acids on protein-synthesizing enzymes is thus not available, since we should have to consider, in addition to the peptidases, at least the enzymes involved in acetyl phosphate production and in amino-acid acetylation.

So far as the peptidases themselves are concerned considerable information on optical specificity has been collected by Bergmann and others. Bergmann, Zervas, Fruton, Schneider & Schleich (1935) showed that yeast dipeptidase hydrolyzed L-leucyl-L-alanine rapidly and L-leucyl-D-alanine at a slower rate. Berger, Johnson & Baumann (1941) found that animal peptidases split D-leucylglycine very much more slowly than the L-isomer, but that the peptidases of *Leuconostoc mesenteroides*, *Clostridium butylicum*, *Bacillus megatherium* and *Pseudomonas fluorescens* hydrolyzed the D-peptide almost as rapidly as the DL-peptide.

It is reasonable to assume, since enzymic reactions are reversible, that these peptidases would also fail to show strict optical specificity in synthetic reactions. In contrast to these enzyme preparations, purified crystalline peptidases have been shown to be optically specific; thus trypsin, chymotrypsin, carboxypeptidase and papain fail to synthesize or hydrolyze peptide bonds formed from D-amino-acids (Bergmann, Zervas & Fruton, 1935; Bergmann & Fraenkel-Conrat, 1937, 1938; Bergmann & Fruton, 1938; Stahmann, Fruton & Bergmann, 1946). A few D-amino-acid peptides and D-amino-acids have been reported to slow the rate of hydrolysis of the corresponding L-peptides by optically specific peptidases (Abderhalden & Abderhalden, 1942), but in some cases this inhibition is believed to be due to formation in solution of a stable racemate (Bergmann & Fruton, 1938; Stahmann *et al.* 1946). Singh & Perti (1945), from a study of camphor β-sulphonic acid, have concluded that a DL-racemate may possess considerable stability in the dissolved state.

Although woefully incomplete, available information on the effect of D-amino-acids on enzymes concerned with protein synthesis thus gives no encouragement to the suggestion that D-amino-acids, as such, are likely to be effective enzyme inhibitors.

In the hope that some further light might be thrown on the relation of optical configuration to antibiotic action, peptides built entirely from L-amino-acids have been compared as growth inhibitors with their isomers containing one 'unnatural' amino-acid (Harris, Work & Fuller, unpubl.). Up to the present no significant difference in antibiotic action between L-L-L-tripeptides and L-D-L-tripeptides has been encountered.

Taken as a whole the above fragments of evidence indicate that antibiotics containing D-amino-acids are active, not because they have this character in common but rather in virtue of their individual structures of which the D-amino-acid must be regarded as an integral structural part.

This view is supported by the evidence of Hotchkiss (1944) and of Gale & Taylor (1946*b*). Although of somewhat similar chemical type to gramicidin-D, and a product of the same micro-organism, tyrocidine seems to act in a completely different manner and by a somewhat non-specific mechanism. Hotchkiss found that gramicidin-D inhibited cell synthesis and possibly phosphorylation without inhibiting cellular oxidation, whereas tyrocidine altered cell permeability and caused loss of nutrients. Gale & Taylor found that in this respect tyrocidine behaved in a similar way to such non-specific bactericidal substances as phenol and several synthetic detergents.

It should be borne in mind that highly specific antibiotics such as gramicidin-D and penicillin probably act by being selectively absorbed

at some enzyme surface, and that this enzyme surface 'fits' a particular arrangement of electrons in space. The contrast between active penicillin synthesized from D-penicillamine and inactive penicillin from L-penicillamine, at first sight an argument in favour of the importance of D-amino-acids, is a contrast between two substances of very different electronic configuration which would show quite different 'fits' towards a highly specific enzyme centre. The optical configuration of any one part of an antibiotic should therefore be regarded as a structural feature of the antibiotic molecule as a whole.

The view that different chemotherapeutic actions of optical isomers are related to the optical form of the enzyme inhibited is by no means new and was discussed by Easson & Stedman in 1933 in relation to the varying pharmacological action of optical isomers. An interesting illustration of this relation has been reported by Russian workers. Some strains of *Bacillus rotans* (*B. mycoides* Flügge) grow in colonies showing a sinistral rotation, others adopt a dextral form (Roberts, 1935). Gause (1942) found that autolysates from sinistral strains of this organism were unable to attack D-leucylglycine, but that an enzyme preparation from the dextral strain easily split the 'unnatural' peptide; in other words, the enzymic systems of the two colonial forms showed structural (i.e. optical) specificity with regard to the molecular species attacked. Recent work has shown that this specificity extends to growth inhibition induced by two optical isomers of mepacrine. Alpatov (1946) concludes that the majority of living organisms, including the L-form of *B. rotans*, are less sensitive to D-mepacrine than to L-mepacrine, but that in dextral *B. rotans* this sensitivity is reversed.

Although optical isomers are generally regarded as extremely closely related to one another, this is by no means the case as far as enzyme inhibition is concerned; for example, Hunter & Downs (1945) have shown that arginase, an enzyme specific for L-arginine, is inhibited by other amino-acids of the L-series, and that the degree of inhibition varies over a wide range according to the nature of the side-chain. D-Amino-acids were completely non-inhibitory and DL-amino-acids gave almost exactly half the inhibition to be expected from the corresponding L-isomers.

In conclusion, attention must be drawn to the fact that several polypeptides have been found to possess specific and powerful enzyme-inhibitory action. Trypsin inhibitor (pancreatic) has an estimated molecular weight of 6000 (not very much larger than gramicidin) and when present in equimolar proportions to trypsin produces complete enzyme inhibition (Kunitz & Northrop, 1936). Figures need not be quoted for enzyme inhibition by chemotherapeutic drugs, but in general it is true to say that far more than an equimolar concentration is required to produce 100% inhibition on those pure enzymes which

have been examined. The only cases of comparable efficacy are inhibitions of SH enzymes, such as urease, by oxidizing agents or heavy metals.

There has been a tendency in the past to regard polypeptides as physiologically inert compounds, but with the discovery of gramicidin-D, gramicidin-S, tyrocidine, subtilin, phalloidin and amanitine as highly toxic molecules; with the demonstration of trypsin inhibitor, soybean trypsin inhibitor, pepsin inhibitor and other polypeptides as highly effective enzyme inhibitors and with the suggestion of the polypeptide nature of the bacterial nutrient strepoginin, and of its antagonist lycomarasmin (Woolley, 1946), peptides can no longer be regarded simply as aggregates of physiologically inert amino-acids, but must be deemed to be a group of compounds offering unlimited possibilities of structural variation and considerable potentialities as pharmacologically specific enzyme inhibitors and chemotherapeutic agents.

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Lysine Analogues as Inhibitors of Bacterial Growth

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Many investigations during recent years have demonstrated that analogues of essential metabolites may exert antibacterial action, but this approach has not so far led to the synthesis of a practical chemotherapeutic agent. It seemed to us that the ubiquity of the amino-acids, coupled with their importance in the biosynthesis of enzymes and other proteins, warranted further investigation of amino-acid analogues and peptides as potential chemotherapeutic agents. As it was impracticable to prepare analogues of every amino-acid, the choice between various alternatives was guided by existing knowledge of amino-acid metabolism.

The majority of amino-acids can undergo rapid deamination and reamination at the α-amino group (Braunstein & Kritzmann, 1937; Schoenheimer, Ratner & Rittenberg, 1939). It seemed undesirable that a potential antimetabolite should be subject to rapid metabolic transformation, and we were, accordingly, faced with the problem of designing a series of amino-acid analogues of such metabolic stability that they might retain the characteristic α-amino-carboxylic acid grouping, during protein synthesis. Experiments with ^{15}N in rats have indicated that lysine was an exception in the general

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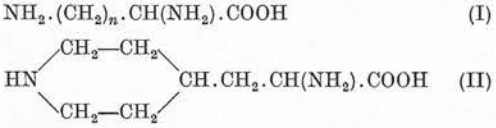
metabolic turnover, and did not receive either of its nitrogen atoms from the nitrogen of other amino-acids or from inorganic nitrogen (Schoenheimer *et al.* 1939). The failure of rats on a lysine-free diet to utilize the α-hydroxy analogue of lysine (McGinty, Lewis & Marvel, 1924) also supports the view that lysine does not participate in transamination reactions in the same way as the majority of amino-acids. This unique metabolic behaviour of lysine suggested that analogues of lysine might possess greater potentialities as antimetabolites than analogues of other amino-acids.

We accordingly synthesized and tested three types of lysine (I, $n=4$) analogue: (1) a higher straight chain homologue (I, $n=5$), 1:6-diaminohexane-1-carboxylic acid; (2) a C–N substituted analogue of lysine (II) in which the terminal amino group and the β-carbon atom of lysine were incorporated into a piperidine ring structure, β-4-piperidyl alanine; and (3) analogues formed by incorporating (II) in amide (peptide) linkage with ammonia or amino-acids.

$$NH_2.(CH_2)_n.CH(NH_2).COOH \qquad (I)$$



(II)

Synthetic methods

Several interesting points arose in the synthesis of the compounds listed in Table 1, and some unexpected difficulties were encountered in attempting to extend the range of compounds under test. Synthesis of 1:6-diaminohexane-1-carboxylic acid was accomplished by an extension of the acetamidomalonic ester synthesis described by Albertson & Archer (1945). Condensation of ethyl acetamidomalonate and 1-chloro-5-benzamidopentane was slow and the isolation of a crystalline salt of homolysine was extremely tedious.

Suitable intermediates for the synthesis of piperidyl-alanines were obtained by condensing α- and γ-picolines with chloral. Whereas 2-(3′:3′:3′-trichloro-2′-hydroxypropyl)-pyridine hydrochloride had previously been obtained in good yield (67 %; Tullock & McElvain, 1939), no satisfactory method existed for the synthesis of the corresponding 4-compound (cf. Alberts & Bachman, 1935). By keeping a mixture of γ-picoline and chloral at 37° for 8 days it was found that 4-(3′:3′:3′-trichloro-2′-hydroxypropyl)pyridine could be obtained in 68 % yield; the best yield previously reported for this compound was 16–18 % (Alberts & Bachman, 1935). Conversion of the trichlorohydroxypropylpyridines to the corresponding pyridylacrylic acids was achieved by the standard method.

Considerable difficulty seems to have been encountered by earlier investigators in attempts to reduce pyridylacrylic acids to the corresponding piperidylpropionic acids; for example, Rabe & Kindler (1919) reduced 4-pyridylacrylic acid by the use of sodium in amyl alcohol. No yields were quoted, but repetition of their method gave disappointing results. Tullock & McElvain (1939) report the failure of Raney nickel under high pressure, a failure which we can also confirm. It was found, however, that 2- and 4-pyridylacrylic acids could be quantitatively reduced at room temperature and atmospheric pressure by use of platinum oxide in the presence of a small excess of hydrochloric acid. In the absence of acid the aromatic ring remains completely unreduced, and in the presence of exactly one equivalent of acid, reduction is slow and incomplete (cf. Rubstov, 1946). The difficulty of catalytic reduction, as was pointed out by Maxted (1948), is probably associated with the existence on the pyridine nitrogen of a free electron pair which leads to the strong adsorption of such a nitrogen atom by a metal catalyst; this self-poisoning effect is eliminated in the presence of hydrochloric acid.

α-Halogenation of *N-benzoyl-2-(4-piperidyl)propionic acid* on a small scale by the method of Eck & Marvel (1934) gave no difficulty, and *N-benzoyl-2-(4′-piperidyl)-1-bromopropionic acid* was isolated in 75 % yield; on a larger scale, however, the reaction with phosphorus and bromine became violently exothermic and in the absence of a suitable solvent the reaction mixture became too viscous to allow effective stirring. The result was incomplete bromination and partial cleavage of the benzamide linkage. α-Chlorination by the method of Galat (1947) using sulphuryl chloride did not give such a good yield as the small-scale bromination with phosphorus and bromine (60 % compared with 75 %), but the reaction was mild and non-exothermic and was accordingly preferred for large-scale work.

Having successfully halogenated benzoyl-2-(4′-piperidyl)propionic acid no difficulty was anticipated in the halogenation of *benzoyl-2-(2′-piperidyl)propionic acid*; nevertheless, we were unable to find any conditions under which the 2-isomer could be α-halogenated without disruption of the *N*-benzoyl-piperidyl radical. The corresponding *2-(p-toluenesulphonyl-2′-piperidyl)propionic acid* was prepared in the hope that the toluenesulphonyl-piperidyl group would be more stable than benzoylpiperidyl, but, under the conditions which had been successful with the 4-isomer, this compound was also degraded and toluenesulphonyl chloride was recovered in almost quantitative yield from the reaction mixture. It will be remembered that von Braun (1904) prepared 1:5-dichloropentane by reaction of benzoylpiperidine with phosphorus pentachloride. It seems probable that some similar reaction may take place with benzoyl-2-piperidylpropionic acid in the presence of bromine and phosphorus.

Conversion of *benzoyl*-2-(4′-*piperidyl*)-1-*chloropropionic* acid to 2-(4′*piperidyl*)-1-*aminopropionic acid* (piperidylalanine) was effected without difficulty. Dicarbobenzyloxypiperidyl-alanine was coupled with leucine methyl ester by the azide method of Bergmann & Zervas (1932). A curious and unexplained abnormality was encountered in this coupling; a considerable quantity of *dicarbobenzyloxy*-2-(4′-*piperidyl*)-1-*aminopropionamide* was formed as a by-product in the conversion of the acid hydrazide to the peptide through the acid azide. The formation of dicarbobenzyloxylysyl amide under similar conditions and in similar yield was reported by Prelog & Wieland (1946), but repeated experiments with other carbobenzyloxy-amino-acid azides did not lead to similar amide formation.

In coupling piperidylalanine with *p*-aminobenzoic acid the acid chloride method was found to be preferable to the azide method; the low basicity of the aromatic amino group of methyl *p*-aminobenzoate prevented rapid reaction between it and the acid azide of dicarbobenzyloxypiperidylalanine.

EXPERIMENTAL

1:6-*Diaminohexane*-1-*carboxylic acid*. To a hot solution of Na (2·3 g.) in dry ethanol (75 ml.) was added ethylacetamidomalonate (31·2 g.) followed by 1-chloro-5-benzamidopentane (17·45 g.). The mixture was boiled for 18 hr., the ethanol removed under reduced pressure, and the oily residue mixed with water. The product was extracted into ether, dried, and the ether removed. The residual oil (24·7 g.) was mixed with aqueous KOH (20%; 10 g.) and heated at 95° for 2 hr.; a water-insoluble oil was removed and the aqueous alkaline solution acidified with excess of HCl; an oil separated which could not be crystallized. Decarboxylation of the substituted malonic acid took place rapidly when the oil was boiled with 6N-HCl and after 10 hr., hydrolysis of the benzamide and acetamido groups was also complete. Benzoic acid was removed from the acid solution which was then concentrated to a thick syrup. The syrup was extracted with ethanol, the insoluble residue discarded and the ethanolic solution of the dihydrochloride concentrated slowly. 1:6-Diaminohexane-1-carboxylic acid could not be crystallized either as the dihydrochloride or as the dipicrate. The diamine-dihydrochloride was mixed with N-HCl, and phosphotungstic acid in N-HCl was added in excess. The solid phosphotungstate was collected, suspended in water and decomposed with baryta. The filtrate and washings from the Ba phosphotungstate were concentrated and left a colourless syrup (4·2 g.). The syrup was dissolved in the minimum volume of water and HCl added to bring the pH to 5·0. 1:6-*Diaminohexane*-1-*carboxylic acid monohydrochloride* crystallized from the solution after cautious addition of ethanol. The product was recrystallized from aqueous ethanol as transparent needles of the monohydrate, m.p. 176° (decomp.). (Found: C, 39·1; H, 8·9; N, 13·1. $C_7H_{16}O_2N_2$.HCl.H_2O requires C, 39·17; H, 8·86; N, 13·05%. Loss in weight at 105°/20 mm., 7·9. $1H_2O$ requires 8·4%.)

4-(3′:3′:3′-*Trichloro*-2′-*hydroxypropyl*)*pyridine* (I). Pure chloral (190 g.; b.p. 97°/755 mm.) and γ-picoline (106 g.) were mixed and kept at 37° for 8 days. The reaction product, a dark brown viscous oil, was diluted with ethanol (500 ml.) and heated three times with successive lots (20 g.) of charcoal. Concentration of the combined filtrates and washings left a light brown solid which crystallized from 50% aqueous methanol as needles, m.p. 160° (210 g.; 68% of theory; cf. Rabe & Kindler, 1919; Alberts & Bachman, 1935).

2-(4′-*Pyridyl*)*acrylic acid* (II). A solution of KOH (67·5 g.) in ethanol (350 ml.) was cooled in ice and an ethanolic solution of (I) (48 g.) was added slowly. The reaction mixture was stirred and the temperature allowed to rise slowly. The mixture was finally heated for 15 min. at 95°, cooled and the precipitated KCl removed. The brown residue remaining after evaporation of the ethanol was dissolved in water and neutralized with acetic acid. The sparingly soluble 2-(4′-pyridyl)acrylic acid (14·5 g.) separated slowly and was collected, m.p. 296°; the hydrochloride crystallized from ethanol as needles, m.p. 244° (cf. Rabe & Kindler, 1919).

2-(4′-*Piperidyl*)*propionic acid* (III). The hydrochloride of (II) (46 g.) in water (300 ml.) containing one equivalent of HCl was hydrogenated at room temperature and atmospheric pressure using Adams's platinum oxide catalyst. Hydrogenation was complete after 60 hr. The catalyst was removed and the solution concentrated. The product was crystallized from hot ethanol. During this procedure some esterification of the piperidylpropionic acid took place and only the first crop (30 g.) of crystalline material which separated on addition of acetone to the ethanolic solution was the hydrochloride of (III), needles, m.p. 244°. (Found: C, 49·8; H, 8·4; N, 7·3. $C_8H_{15}O_2N$.HCl requires C, 49·6; H, 8·3; N, 7·2%.) Addition of ether to the mother liquor of the acid hydrochloride gave a second crop of crystalline material (11 g.), m.p. 129–130°, which was identified as the hydrochloride of *ethyl*-2-(4′-*piperidyl*)*propionate*. (Found: C, 54·4; H, 9·3; N, 6·2. $C_{10}H_{19}O_2N$.HCl requires C, 54·2; H, 9·0; N, 6·3%.)

Benzoyl 2-(4′-*piperidyl*)*propionic acid* (IV). Benzoylation was carried out at 0° by dropwise addition of benzoyl chloride (15 g.) to an alkaline solution (50 ml. 2N-NaOH) of the hydrochloride of (III) (19 g.). A further equivalent of alkali (25 ml., 2N) was added during the period of benzoylation. The product separated on acidification of the reaction mixture and was crystallized from ethanol as white prisms, m.p. 148° (21·8 g.). (Found: C, 69·2; H, 7·4; N, 5·4. $C_{15}H_{19}O_3$ requires C, 69·0; H, 7·3; N, 5·4%.)

Benzoyl 2-(4′-*piperpidyl*)-1-*bromopropionic acid* (V). The method of bromination was essentially that of Eck & Marvel (1934). Benzoyl-2-(4′-piperidyl)propionic acid (4·9 g.) was mixed with red P (0·83 g.) and allowed to react at 0° with Br_2 (12 g.). The reaction was completed by heating and the α-bromo acid (5·0 g., 78% theory) was isolated by the method recommended by Eck & Marvel. After repeated crystallization from ethanol the product (3·4 g.) was obtained in the form of white plates melting at 183–184°. (Found: C, 53·3; H, 5·5; Br, 23·2. $C_{15}H_{18}O_3NBr$ requires C, 53·0; H, 5·3; Br, 23·5%.)

Benzoyl 2-(4′-*piperidyl*)-1-*chloropropionic acid* (VI). Chlorination was effected by the method of Galat (1947). Benzoyl 2-(4′-piperidyl) propionic acid (30 g.) was intimately mixed with powdered I_2 (0·5 g.) and the mixture added to SO_2Cl_2 (70 ml.). The reaction mixture was heated at 60–65° for 2 hr. and then gradually brought to the boil to complete the reaction. Sulphuryl chloride was removed under reduced pressure and the viscous residue poured into a mixture of ice and water. The material which separated gradually solidified.

The solid (33·5 g.) was ground to a fine powder, washed thoroughly, dried and dissolved in hot ethanol. The product crystallized from ethanol as pale yellow prisms (16 g.), m.p. 170°. The mother liquors were reworked by evaporation to dryness, trituration with benzene and crystallization of the benzene-insoluble residue from 80% ethanol. The combined crops of crystalline material (24 g.) were recrystallized from ethanol, yield 20 g. (60% theory), m.p. 174°. (Found: C, 60·6; H, 6·1; Cl, 12·4. $C_{15}H_{18}O_3NCl$ requires C, 60·9; H, 6·1; Cl, 12·0%.)

2-(*Benzoyl-4′-piperidyl*)-1-*aminopropionic acid* (VII). The chloroacid (VI) (11 g.) was mixed with NH_3 (175 ml., *d* 0·88) and heated under pressure for 12 hr. at 100°. Excess of ammonia was removed under reduced pressure and Cl^- eliminated by addition of Ag_2SO_4. Excess Ag was removed as the sulphide and SO_4 as $BaSO_4$. The product was a crystalline solid (7·5 g.), m.p. 216–218°, which was used for the next stage without further purification.

2-(4′-*piperidyl*)-1-*aminopropionic acid* (VIII). The product of the previous experiment (6·5 g.) was debenzoylated by boiling for 10 hr. with 6 N-HCl (120 ml.). The acid solution was freed from benzoic acid and concentrated to dryness. The residue was crystallized by the careful addition of ether to an ethanolic solution of the dihydrochloride containing a few drops of water. The pure dihydrochloride monohydrate (4·2 g.) separated from aqueous acetone or from a mixture of ethanol and ether as plates, m.p. 160°. (Found: C, 36·5; H, 7·6; N, 10·4. $C_8H_{16}O_2N_2.2HCl.H_2O$ requires C, 36·5; H, 7·6; N, 10·6%. Loss of weight at 120°, 6·6%. $1H_2O$ requires 6·8%. Anhydrous dihydrochloride: N, 11·6. $C_8H_{16}O_2N_2.2HCl$ requires N, 11·4%.)

Dicarbobenzyloxy-2-(4′-*piperidyl*)-1-*aminopropionic acid* (IX). The dicarbobenzyloxy derivative was prepared according to the method of Bergmann, Zervas & Ross (1935). From 4 g. of 2-(4′-piperidyl)-1-aminopropionic acid dihydrochloride we obtained 6·2 g. of the dicarbobenzyloxy derivative as a colourless syrup which could not be crystallized.

Dicarbobenzyloxy - 2 - (4′-*piperidyl*) - 1 - *aminopropionamide* (X). The product (IX) from the above experiment was esterified by treatment with excess diazomethane. A portion of the ester (1·8 g.) was mixed with excess ammoniacal methanol (saturated at 0°) and heated under pressure at 37° for 48 hr. The solvent was removed and the product crystallized from ethanol, prisms, m.p. 134° (1·4 g.). (Found: C, 65·4; H, 6·5; N, 9·8. $C_{24}H_{29}O_5N_3$ requires C, 65·6; H, 6·6; N, 9·6%.)

2 - (4′ - *Piperidyl*) - 1 - *aminopropionamide dihydrochloride* (XI). Compound (X) (1·1 g.) in methanol (30 ml.) containing 2 equiv. acid (0·18 ml. of 10 N-HCl) was hydrogenated in the presence of palladium black (300 mg.). When evolution of CO_2 was complete (3 hr.) the catalyst was removed, the solvent evaporated, and the residual amide dihydrochloride (0·5 g.) crystallized as plates from a mixture of methanol and acetone, m.p. 292° (decomp.). (Found: C, 39·1; H, 7·9; N, 17·5. $C_8H_{17}ON_3.2HCl$ requires C, 39·3; H, 7·8; N, 17·2%.)

Dicarbobenzyloxy - 2 - (4′ - *piperidyl*) - 1 - *aminopropionic acid hydrazide* (XII). Excess of hydrazine (1 ml., 90%) was added to a solution of dicarbobenzyloxy 2-(4′-piperidyl)-1-aminopropionic acid methyl ester (2 g.) in methanol (20 ml.). After 24 hr. at room temperature the solvent was removed from the mixture and the residual oil crystallized from a mixture of ethyl acetate and ligroin. The pure hydrazide (1·8 g.) separated as plates, m.p. 115°. (Found: C, 63·7; H, 6·6; N, 12·3. $C_{24}H_{30}O_5N_4$ requires C, 63·4; H, 6·6; N, 12·3%.)

Dicarbobenzyloxy - 2 - (4′ - *piperidyl*) - 1 - *aminopropionyl*-DL-*leucine methyl ester* (XIII). A. *Azide method.* The hydrazide (XII) (1·6 g.) was dissolved in glacial acetic acid (5 ml.) and diluted with N-HCl (15 ml.). The mixture was cooled to 0° and a cooled solution of $NaNO_2$ (1·1 g.) in water (10 ml.) was added dropwise to the stirred solution. The precipitated acid azide was extracted into ethyl acetate (50 ml.) and the ethyl acetate washed successively at 0° with pre-cooled water, with cooled saturated $NaHCO_3$ solution and again with water. The neutral solution of azide in ethyl acetate was quickly dried over Na_2SO_4 and added during 10 min. to a cooled solution of leucine methyl ester (0·5 g.) in ether (25 ml.). The mixture was left for 24 hr. and then extracted with N-HCl. Excess leucine ester was recovered from the aqueous acid solution. The organic solvent mixture was then washed with $NaHCO_3$, with water, and dried over Na_2SO_4. Removal of solvent *in vacuo* left a pale yellow gum (0·6 g.) which crystallized after trituration with ethyl acetate. The crystalline product (0·2 g.) was not the dipeptide but dicarbobenzyloxy-2-(4′-piperidyl)-1-aminopropionamide; plates, m.p. 132–133°. (Found: C, 65·9; H 6·6; N, 9·7. $C_{24}H_{29}O_5N_3$ requires C, 65·6; H, 6·6; N, 9·6%.) The required dipeptide, m.p. 104° (0·25 g.), was isolated from the ethyl acetate mother liquors of the amide by addition of light petroleum. (Found: C, 65·8; H, 7·4; N, 7·6. $C_{31}H_{41}O_7N_3$ requires C, 65·6; H, 7·2; N, 7·4%.)

B. *Acid chloride method.* As an alternative to the above method the same peptide was prepared by the conversion of the acid (IX) (1·0 g.) to the acid chloride by treatment with PCl_5 (0·5 g.) in ether (20 ml.) at 0°. When the PCl_5 had entirely dissolved, the solution was filtered and diluted with light petroleum. The solvent was decanted from the precipitated acid chloride which was immediately dissolved in ether, washed quickly in ice-cold water (10 ml.) and dried over Na_2SO_4. DL-Leucine methyl ester hydrochloride (1·5 g.) was dissolved in water (10 ml.) and the aqueous solution covered with ether (20 ml.). The mixture was cooled to −10° and the amino-acid ester forced into the ether layer by gradual addition of a large excess of anhydrous K_2CO_3. The ether layer was decanted from the K_2CO_3 sludge, quickly dried over Na_2SO_4 and filtered. The acid chloride solution already prepared was added dropwise, with shaking, to 2 equiv. of the ester solution, and after 1 hr. the leucine methyl ester hydrochloride which had separated was removed by filtration. The ethereal filtrate was washed successively with water, N-HCl, $KHCO_3$ (7%) and water, dried and concentrated *in vacuo*. The product crystallized from a mixture of ethyl acetate and light petroleum as platelets, m.p. 104–106° (0·25 g.) (Found: C, 65·9; H, 7·4; N, 7·8. $C_{31}H_{41}O_7N_3$ requires C, 65·6; H, 7·2; N, 7·4%.)

2-(4′-*Piperidyl*)-1-*aminopropionyl*-DL-*leucine methyl ester* (XIV). The carbobenzyloxy groups were removed from (XIII) (0·15 g.) by catalytic hydrogenation by the same method as was used in (XI). The dihydrochloride was hygroscopic and could not be crystallized. As removal of carbobenzyloxy groups by this method is essentially quantitative, the product was deemed pure enough for test without crystallization.

Dicarbobenzyloxy - 2 - (4′ - *piperidyl*) - 1 - *aminopropionyl* - *p-aminobenzoic acid methyl ester* (XV). An ethereal solution of the acid chloride of (IX) prepared as already described from (IX) (1·0 g.) was added dropwise, with shaking, to a solution of 2 equiv. (0·7 g.) of methyl-*p*-aminobenzoate in dry $CHCl_3$ (20 ml.). After leaving for 18 hr. at 37°, crystalline methyl-*p*-aminobenzoate hydrochloride (0·38 g.) was re-

moved and the $CHCl_3$-ether solution washed successively with dilute acid, bicarbonate and water. Removal of the solvent left a syrup (0·8 g.) which was redissolved in ethyl acetate and boiled with activated charcoal. The product, a colourless viscous oil (0·5 g.), could not be crystallized and was used for the next stage without further purification.

2 - (4′ - *Piperidyl*) - 1 - *aminopropionyl* - p - *aminobenzoic acid* (XVI). The acylated peptide ester (XV) (0·5 g.) was hydrolysed in a mixture of dioxane (5 ml.) and N-NaOH (5 ml.). After 30 min. at room temperature the mixture was acidified and diluted with water; the oil which separated was extracted into $CHCl_3$. The $CHCl_3$ was removed *in vacuo* and the residue hydrogenated in the presence of palladium black (100 mg.) and 2 equiv. of HCl. The dihydrochloride dihydrate of (XVI) crystallized as needles (0·1 g.), m.p. 190–192° (decomp.), when ether was added slowly to a methanolic solution of the product of hydrogenation. (Found: C, 45·0; H, 7·0; N, 10·7. $C_{15}H_{21}O_3N_3.2HCl.2H_2O$ requires C, 45·0; H, 6·8; N, 10·5%. Loss in weight at 105°, 8·4%. $2H_2O$ requires 9·0%.)

2 - (3′ : 3′ : 3′ : - *Trichloro* - 2′ - *hydroxypropyl*)*pyridine hydrochloride* (XVII). The method used was based on earlier methods described by Einhorn & Liebrecht (1887), Löffler & Kaim (1909) and Tullock & McElvain (1939). Anhydrous chloral (110 ml.) was mixed with α-picoline (130 ml.) and dry amyl acetate (200 ml.). The mixture was heated for 14 hr. at 130° and then steam distilled to remove solvent and excess α-picoline. The non-volatile residue was decolorized with charcoal and concentrated *in vacuo*. After drying thoroughly over H_2SO_4, the product was crystallized from ethanol as needles, m.p. 200–201° (120 g.) (cf. Tullock & McElvain, 1939).

2-(2′-*Piperidyl*)*propionic acid hydrochloride* (XVIII). 2-(2′-Pyridyl)acrylic acid was prepared by the method of Löffler & Kaim (1909); the acrylic acid was reduced by the method used for the isomeric acid (III) and the product (XVIII) crystallized from aqueous acetone as the hydrochloride, m.p. 191–192°. The yield in the reduction was almost quantitative and gave none of the difficulties reported for other methods (Tullock & McElvain, 1939; Löffler & Kaim, 1909). (Found: C, 49·5; H, 8·3; N, 7·1. $C_8H_{15}O_2N.HCl$ requires C, 49·6; H, 8·3; N, 7·2%.)

Benzoyl-2-(2′-*piperidyl*)*propionic acid* (XIX). The method used for the isomeric compound (IV) was applied successfully to (XVIII) and the product crystallized from ethanol as needles, m.p. 158°. (Found: C, 69·0; H, 7·3; N, 5·7. $C_{15}H_{19}O_3N$ requires C, 69·0; H, 7·3; N, 5·4%.)

p-*Toluenesulphonyl*-2-(2′-*piperidyl*)*propionic acid* (XX). Tosylation was effected by the method of Thomas & Goerne (1919). A solution of (XVIII) (2 g.) in N-NaOH (25 ml.) was shaken vigorously with *p*-toluenesulphonyl chloride (2·5 g.) for 3 hr. Unchanged acid chloride was removed by filtration and the filtrate acidified. The precipitate was crystallized from 80% ethanol, prisms, m.p. 108–109° (1·0 g.) (Found: C, 57·6; H, 7·0; N, 4·7. $C_{15}H_{21}O_4NS$ requires C, 57·9; H, 6·8; N, 4·5%.)

MICROBIOLOGICAL RESULTS

When tested *in vitro* against *Streptococcus haemolyticus* (in Hartley's digest broth), *Staphylococcus aureus* (in medium of Strauss, Dingle & Finland, 1941) and *Escherichia coli* (in medium of MacLeod, 1940, supplemented with casein), analogues of lysine were found to be slightly antibacterial. Furthermore, the antibacterial activity of a simple analogue (β-4-piperidylalanine) was enhanced by its incorporation in peptide linkage with other amino-acids as indicated in Table 1.

Table 1. *Minimum inhibitory concentrations* (M) *of lysine analogues as inhibitors of bacterial growth*

	Haemolytic streptococci		*Staph. aureus*		*Esch. coli*	
Compound	Blood	Broth	Broth	Synthetic	Broth	Synthetic
1:6-Diaminohexane-1-carboxylic acid mono HCl	—	10^{-3}	$>10^{-2}$	—	$>10^{-2}$	—
2(4′-Piperidyl)-1-aminopropionic acid dihydrochloride	2×10^{-2}	8×10^{-3}	$>4\times10^{-2}$	2×10^{-2}	$>4\times10^{-2}$	$>4\times10^{-2}$
2(4′-Piperidyl)-1-aminopropionamide dihydrochloride	—	4×10^{-3}	$>4\times10^{-3}$	—	$>4\times10^{-3}$	—
2(4′-Piperidyl)-1-aminopropionyl-*p*-aminobenzoic acid dihydrochloride	—	$1{\cdot}5\times10^{-3}$	$>6\times10^{-3}$	6×10^{-3}	$>6\times10^{-3}$	$>6\times10^{-3}$
2(4′-Piperidyl)-1-aminopropionyl-DL-leucine methyl ester dihydrochloride	—	$1{\cdot}5\times10^{-3}$	$>1{\cdot}5\times10^{-3}$	—	$>1{\cdot}5\times10^{-3}$	—

DISCUSSION

At the time (1946) when the present investigation was planned some analogues of amino-acids were already known to exhibit limited antibacterial action: thus Fildes (1941) showed that indole-acrylic acid, an analogue of tryptophan, inhibited growth of *Esch. coli* at a concentration of 8×10^{-3}M; the effect was reversed by tryptophan. McIlwain (1941) had shown that α-aminosulphonic acids had some growth inhibitory action; Harris & Kohn (1941) had reported inhibition of growth of *Esch. coli* by ethionine; Roblin, Lampen, English, Cole & Vaughan (1945) had found that methoxinine, the oxygen analogue of methionine, inhibited *Esch. coli* and *Staph. aureus* in synthetic media; du Vigneaud, McKennis, Simmonds, Dittmer & Brown (1945) had shown that β-2-thienyl-alanine acted as a phenyl-

alanine antagonist, and Waelsh, Owades, Miller & Borek (1946) had studied methionine sulphoxide, methionine sulphone and benzylhomocysteine sulphoxide as antimetabolites of glutamic acid.

While the present investigation was under way the published knowledge of amino-acid analogues as growth inhibitors was augmented by a paper by Mitchell & Nieman (1947) on halogenated phenylalanines and tyrosines, of which 3-fluoro-DL-phenylalanine and 3-fluoro-L-tyrosine were the most active; by a communication from Elks, Hems & Ryman (1948) on α-amino acids with longer than normal aliphatic chains which were ineffective as growth inhibitors, and by a paper by Elliott, Fuller & Harington (1948) on alanines substituted in the β-position with pyridine, quinoline and basic derivatives of benzene. Significant inhibitions were observed only with β-ω-amino-*p*-tolylalanine, β-6-methoxyquinolyl-4-alanine and β-pyridyl-4-alanine; these compounds inhibited growth of *Strep. pyogenes* in broth at concentrations of 2×10^{-3}, $1{\cdot}5 \times 10^{-3}$ and 8×10^{-3} M respectively. Other substituted alanines showing significant action as inhibitors of bacterial growth were β-2-furylalanine and β-2-pyrrolealanine (Clark & Dittmer, 1948; Herz, Dittmer, and Cristol, 1948).

Without comparative tests, using the same strain of organism and the same batch of culture medium, it is impossible to make any comparison in absolute terms between the antibacterial efficacy of amino-acid analogues prepared in different laboratories. If any conclusion can be drawn from the experimental evidence available, it is that, as possible chemotherapeutic agents, essential amino-acid analogues do not offer great promise. The results obtained in the present investigation (Table 1) indicate, however, that in our series antibacterial activity was increased when an analogue of lysine (β-4-piperidyl alanine) was combined in peptide linkage with other amino-acids. This led to the abandonment of the present work, and to a study of synthetic peptides as possible antibacterial compounds; the results obtained will be reported in future papers.

SUMMARY

1. The reasons for synthesizing analogues of lysine as potential chemotherapeutic agents are discussed.

2. The following analogues have been synthesized and tested as inhibitors of bacterial growth against *Streptococcus haemolyticus*, *Staphylococcus aureus* and *Escherichia coli*: (1) 1:6-diaminohexane-1-carboxylic acid (homolysine); (2) 2-(4′-piperidyl)-1-aminopropionic acid (β-4-piperidyl alanine); (3) 2-(4′-piperidyl)-1-aminopropionyl-*p*-aminobenzoic acid; (4) 2-(4′-piperidyl)-1-aminopropionyl-DL-leucine methyl ester.

3. No striking growth inhibitory properties were encountered but increased inhibitory action was apparent when (2) was incorporated in peptide linkage with *p*-aminobenzoic acid and DL-leucine.

We are indebted to Dr A. T. Fuller for the biological results given in Table 1.

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The Synthesis of Peptides Related to Gramicidin S and the Significance of Optical Configuration in Antibiotic Peptides

1. TRIPEPTIDES

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In a previous communication (Harris & Work, 1950) we reported that an amino-acid analogue which was not itself antibacterial might acquire antibacterial properties when joined in peptide linkage with other amino-acids.

Repeated demonstration of the occurrence of amino-acids of 'unnatural' (D) configuration in antibiotic polypeptides such as gramicidin (Lipmann, Hotchkiss & Dubos, 1941), tyrocidine (Gordon, Martin & Synge, 1943), gramicidin S (Synge, 1945), aerosporin (Jones, 1948), and bacitracin (Barry, Gregory & Craig, 1948), suggested that antibiotic activity might be dependent upon the occurrence in such peptides of amino-acid analogues (viz. D-amino-acids) which, although harmless in themselves, became antibacterial when incorporated in a suitable peptide sequence. The presence of D-penicillamine among the hydrolysis products of penicillin, and the subsequent demonstration by du Vigneaud, Carpenter, Holley, Livermore & Rachele (1946) that antibiotic penicillin could be synthesized from D-penicillamine, whereas the isomeric compound from L-penicillamine was inactive, seemed at first sight to lend support to this view.

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To test such a theory it did not seem sufficient to link any one of the D-amino-acids occurring in antibiotic peptides with a random selection of L-amino-acids, and the choice of a suitable series of synthetic peptides was governed by the knowledge that in only one antibiotic peptide, namely, gramicidin S, was the probable sequence of amino-acids known.

On the basis of degradative studies, Consden, Gordon, Martin & Synge (1947) have suggested that gramicidin S is a cyclic pentapeptide or a cyclic decapeptide with the following sequence of amino-acid residues: L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl-L-valyl- (Fig. 1). As the only amino-acid of 'unnatural' configuration, phenylalanine is indicated in Fig. 1 by the projection of the benzyl side chain below the line of the peptide 'backbone'. We sought to examine the significance of optical configuration by synthesis, in the first instance, of the tripeptide fragment enclosed by the dotted lines in Fig. 1. The corresponding tripeptide with all three amino-acids in the L configuration was also synthesized as a control.

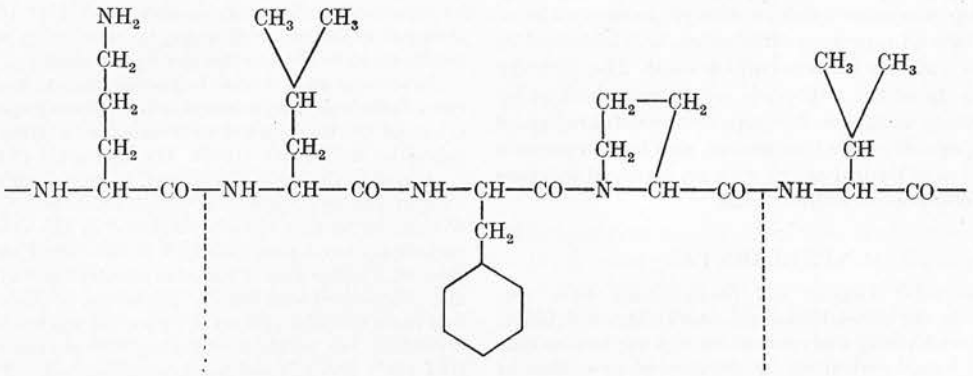

Fig. 1. Amino-acid sequence in gramicidin S.

Synthetic methods

For the syntheses of the optically isomeric tripeptides, carbobenzyloxy derivatives of appropriate amino-acids were used according to the general pro-

cedure of Bergmann & Zervas (1932). Difficulties were encountered in the syntheses of dipeptide esters containing proline, and although *carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester* and *carbobenzyloxy-D-phenylalanyl-L-proline methyl ester* were obtained by the azide method, yields were low and the products were not crystallized. It is relevant to note that Synge (1948) also reported difficulty in the isolation of pure dipeptides containing proline; his products were amorphous and analytical figures departed appreciably from the expected values.

In view of the tendency of proline dipeptides to form diketopiperazines (Abderhalden & Nienburg, 1933; Smith & Bergmann, 1944), and as satisfactory characterization of intermediates was considered desirable, it was decided to modify the order of coupling. In the preparation of carbobenzyloxy-L-leucyl-D-phenylalanine ester the acid chloride and azide methods of coupling were compared; a higher yield and a purer product was obtained by the azide method and this was accordingly the method of choice whenever applicable. The azide from carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide coupled smoothly with L-proline methyl ester to give *carbobenzyloxy - L - leucyl - D - phenylalanyl - L - proline methyl ester* in 85% yield; the corresponding carbobenzyloxy-L-L-L-tripeptide ester, prepared by coupling carbobenzyloxy-L-leucyl azide with L-phenylalanyl-L-proline ethyl ester, was obtained in inferior yield as an amorphous solid. The identity and purity of the tripeptide esters was checked by elementary analyses, by paper chromatography of the tripeptide esters themselves, and by comparison of their acid hydrolysates with an artificial mixture of the component amino-acids.

EXPERIMENTAL

Amino-acids. Leucine and phenylalanine were synthesized by the acetamidomalonic ester synthesis of Albertson & Archer (1945), and resolved through the brucine salts of their formyl derivatives by the classical procedures of Fischer (1906). L-Proline was isolated from a protein hydrolysate by the rhodanilate method of Bergmann (1935).

Carbobenzyloxy-L-phenylalanyl hydrazide. L-Phenylalanine methyl ester was prepared according to the general method of Fischer (1901). The methyl ester (13·0 g.) in dry ether (100 ml.) was cooled to $-10°$ and an ethereal solution of carbobenzyloxy chloride (6·2 g., 0·5 equiv.) was added dropwise, with stirring, during 45 min. The reaction mixture was left overnight at room temperature and the precipitated L-phenylalanine methyl ester hydrochloride recovered by filtration. Carbobenzyloxy-L-phenylalanine methyl ester was obtained as an oil (10·0 g.) after removal of solvent from the filtrate. This oil was mixed in methanol with an excess of 50% hydrazine hydrate and left for 24 hr. at room temperature. *Carbobenzyloxy-L-phenylalanine hydrazide* was precipitated on addition of water and collected by filtration. The pure hydrazide (7·9 g.) was obtained as needles, m.p. 168°, from methanol. (Found: C, 65·4; H, 6·2; N, 13·6. $C_{17}H_{19}O_3N_3$ requires C, 65·2; H, 6·1; N, 13·4%.)

Carbobenzyloxy-D-phenylalanine hydrazide. D-Phenylalanine hydrochloride (25 g.) yielded carbobenzyloxy-D-phenylalanine methyl ester (20 g.). The ester was converted to *carbobenzyloxy-D-phenylalanine hydrazide* by the method previously used for the L-isomer. The hydrazide crystallized from ethanol as needles, m.p. 169–170° (16·5 g.). (Found: C, 65·6; H, 6·4; N, 13·5. $C_{17}H_{19}O_3N_3$ requires C, 65·2; H, 6·1; N, 13·4%.)

Carbobenzyloxy - L - phenylalanyl - L - proline ethyl ester. L-Phenylalanine hydrazide (6·7 g.) in a mixture of glacial acetic acid (50 ml.) and 2N-HCl (100 ml.) was cooled to 0° and a solution of $NaNO_2$ (1·1 g.) in water (10 ml.) added dropwise to the stirred mixture during 10 min. The precipitated azide was extracted into cold ether (100 ml.) and washed with ice-cold water, with cold saturated $NaHCO_3$ solution and again with water. The azide solution was dried quickly over Na_2SO_4 and added during 20 min. to a cooled solution of L-proline ethyl ester (4·0 g.) in dry ether (100 ml.). After 24 hr. at 0° the solution was washed with dilute acid to remove proline ester, then with $NaHCO_3$ solution, and finally with water. Removal of the solvent *in vacuo* left a pale yellow solid (8·0 g.) which was not obtained in a crystalline form. (Found: C, 67·5; H, 6·3; N, 6·8. $C_{24}H_{28}O_5N_2$ requires C, 67·9; H, 6·6; N, 6·6%.)

L-Phenylalanyl-L-proline ethyl ester. Removal of the carbobenzyloxy group from carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester was readily accomplished by application of the catalytic hydrogenation procedure of Bergmann & Zervas (1932). *L-Phenylalanyl-L-proline ethyl ester hydrochloride* crystallized from ethanol as extremely hygroscopic needles, m.p. (sealed tube) 174° (decomp.). Owing to rapid uptake of water, no satisfactory analytical results could be obtained for this hydrochloride.

Carbobenzyloxy - L - leucyl - L - phenylalanyl - L - proline ethyl ester. Carbobenzyloxy-L-leucyl hydrazide was prepared from L-leucine by the method of Bergmann, Zervas, Fruton, Schneider & Schleich (1935). The hydrazide (2·0 g.) was converted to the azide and allowed to react with L-phenylalanyl-L-proline ethyl ester (2·7 g.). The method of handling was similar to that already employed in the synthesis of carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester. The product, a yellow gum (1·9 g.), was dissolved in ethyl acetate and precipitated with ligroin. Repetition of this operation four times yielded a product (1·5 g.) which was not obviously crystalline but which nevertheless melted rather sharply (134–136°). Analysis and paper chromatography of a hydrolysate left no doubt that it was *carbobenzyloxy-L-leucyl-L-phenylalanyl-L-proline ethyl ester.* (Found: C, 67·2; H, 7·3; N, 8·0. $C_{30}H_{39}O_6N_3$ requires C, 67·1; H, 7·3; N, 7·8%.)

L-Leucyl-L-phenylalanyl-L-proline ethyl ester. The carbobenzyloxy derivative (1·2 g.) from the previous experiment was successfully hydrogenated in ethanol in the presence of 1 equiv. of H_2SO_4. Removal of acid and solvent left an oil (0·95 g.) which could not be crystallized. Provisional identification of the product as *L-leucyl-L-phenylalanyl-L-proline ethyl ester* was achieved by paper chromatography.

Carbobenzyloxy-L-leucyl-D-phenylalanine ethyl ester. (*Acid chloride method.*) Carbobenzyloxy-L-leucine (6·2 g.) in dry ether (30 ml.) was allowed to react at 0° with PCl_5 (4·9 g.). When the reaction was complete, ether was removed and the residual oil washed with three 50 ml. portions of dry light petroleum. The petroleum-insoluble acid chloride was dissolved in dry $CHCl_3$ (25 ml.) and half of this solution was added, with stirring, to a cooled solution ($-10°$) of D-phenylalanine ethyl ester (4·5 g.) in dry $CHCl_3$ (25 ml.); after 5 min.

$KHCO_3$ (2·32 g.) was added in the minimum volume of water and followed by the remainder of the acid chloride. The mixture was stirred and the temperature held below 0° for 30 min. and at 15° for another hour. The $CHCl_3$ solution was washed successively with dilute acid and dilute bicarbonate and dried. Removal of solvent left carbobenzyloxy-L-leucyl-D-phenylalanine ethyl ester which crystallized from a mixture of ethyl acetate and light petroleum as needles (5·0 g.), m.p. 105°. (cf. Synge, 1948). (Found: C, 67·8; H, 7·2; N, 6·2. $C_{25}N_{32}O_5N_2$ requires C, 68·1; H, 7·3; N, 6·4%.)

L-Leucyl-D-phenylalanine ethyl ester. The carbobenzyloxy peptide as prepared above (1·0 g.) was hydrogenated in methanol containing 1 equiv. HCl. The carbobenzyloxy group was readily removed and *L-leucyl-D-phenylalanine ethyl ester hydrochloride* crystallized from ethyl acetate-ethanol as plates (0·8 g.), m.p. 216°. (Found: C, 59·6; H, 7·7; N, 8·0. $C_{17}H_{26}O_3N_2$.HCl requires C, 59·6; H, 7·9; N, 8·2%.)

Carbobenzyloxy - L - leucyl - D - phenylalanine methyl ester. (*Azide method.*) Carbobenzyloxy-L-leucyl hydrazide (18 g.) was converted to the azide and allowed to react with phenylalanine methyl ester (16·8 g.). The method was essentially that employed already in the synthesis of carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester. The product, *carbobenzyloxy-L-leucyl-D-phenylalanine methyl ester* (20·8 g.) crystallized as needles, m.p. 110–112°, from ether. (Found: C, 67·4; H, 7·1; N, 6·7. $C_{24}H_{30}O_5N_2$ requires C, 67·6; H, 7·1; N, 6·6%.) Comparison of this yield (77%) with that obtained by the acid chloride method (48%) indicated that the azide method was to be preferred.

Carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide. The methyl ester from the above preparation (19·0 g.) was converted to the hydrazide by treatment in methanol, with an excess of 90% hydrazine hydrate. *Carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide* (16·6 g.) crystallized from ethyl acetate as needles, m.p. 169. (Found: C, 64·5; H, 7·2; N, 13·2. $C_{23}H_{30}O_4N_4$ requires C, 64·8; H, 7·1; N, 13·2%.)

Carbobenzyloxy-L-leucyl-D-phenylalanyl-L-proline methyl ester. Carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide (16·0 g.) was converted to azide and reacted with proline methyl ester (12 g.) by the usual azide method, the only modifications being substitution of ethyl acetate as solvent in place of ether and allowance of 48 hr. at 0° for completion of the reaction. Excess proline ester was recovered as the hydrochloride (6·6 g.). *Carbobenzyloxy-L-leucyl-D-phenylalanyl-L-proline methyl ester* (19·0 g.) was a solid of doubtful crystallinity melting over the range 40–42° ($[\alpha]_D^{22°}$, −42·2° in methanol, *c*, 2·7). Comparison of an acid hydrolysate of this peptide with an artificial mixture of the constituent amino-acids on a paper chromatogram, coupled with almost quantitative reduction of a sample of carbobenzyloxy tripeptide ester to a crystalline tripeptide ester hydrochloride, left no doubt that the desired product had been obtained in an almost pure state in a yield of 85% of theory; analysis of the amorphous solid confirmed this view. (Found: C, 65·9; H, 7·1; N, 8·5. $C_{29}H_{37}O_6N_3$ requires C, 66·5; H, 7·1; N, 8·1%.)

L-Leucyl-D-phenylalanyl-L-proline methyl ester. The carbobenzyloxy tripeptide ester (1·6 g.) from the previous experiment was hydrogenated with palladium black in methanol in the presence of 1 equiv. of HCl. Evolution of CO_2 was complete in 2 hr. *L-Leucyl-D-phenylalanyl-L-proline methyl ester hydrochloride*, white hygroscopic plates, m.p. 240° (decomp.), crystallized from a mixture of ethanol and ether; $[\alpha]_D^{20°}$ − 38·9° in methanol (*c*, 2·0). (Found: C, 58·9; H, 7·5; N, 10·0. $C_{21}H_{31}O_4N_3$.HCl requires C, 59·2; H, 7·5; N, 9·9%.)

MICROBIOLOGICAL RESULTS

The optically isomeric tripeptide ester hydrochlorides, together with the intermediate dipeptide esters, were tested *in vitro* against *Staphylococcus aureus*, *Streptococcus haemolyticus* and *Escherichia coli* (media: see Harris & Work, 1950), and the results obtained are given in Table 1. It was not considered necessary to remove the ester group before testing since esters are readily hydrolysed by living cells.

Table 1. *Minimum inhibitory concentrations* (M) *of synthetic peptide esters as inhibitors of bacterial growth*

Compound	*Esch. coli* (synthetic medium)	*Staph. aureus* (synthetic medium)	*Strep. haemolyticus* (broth)
D-Phenylalanyl-L-proline methyl ester	3×10^{-2}	2×10^{-2}	3×10^{-3}
L-Phenylalanyl-L-proline ethyl ester	2×10^{-2}	4×10^{-2}	$1{\cdot}5 \times 10^{-2}$
L-Leucyl-D-phenylalanine methyl ester	5×10^{-3}	2×10^{-3}	1×10^{-3}
L-Leucyl-D-phenylalanyl-L-proline methyl ester	7×10^{-3}	5×10^{-3}	$1{\cdot}5 \times 10^{-3}$
L-Leucyl-L-phenylalanyl-L-proline ethyl ester	$>7 \times 10^{-3}$	6×10^{-3}	1×10^{-3}

DISCUSSION

It is apparent from Table 1 that synthetic L-leucyl-D-phenyl-alanyl-L-proline ester is slightly antibacterial, but this activity is not peculiarly associated with the occurrence of D configuration, since the optically isomeric L-leucyl-L-phenylalanyl-L-proline ester is equally active.

This result is in general agreement with the findings of other investigators which have been published while the present work was in progress. In view of the isolation of D-leucine and D-valine from gramicidin (Dubos) hydrolysates, and since the intact gramicidin molecule contains no free primary amino groups, Fling, Minard & Fox (1947) synthesized prolyl derivatives of leucine and valine. No antipodal specificity was encountered despite the fact that the free D-amino-acids had shown some inhibition of bacterial growth under similar conditions (Fox, Fling & Bollenback, 1944; Fling & Fox, 1945). All four diastereoisomeric leucylleucines have also been synthesized and tested, but not one had antibacterial activity equal to that of D-leucine itself (Fox, Kobayashi, Melvin & Minard, 1948). The high content of D-leucine and L-tryptophan in gramicidin

led Fruton (1948) to synthesize the diketopiperazine from D-leucine and L-tryptophan, but no antibacterial activity was encountered.

These findings indicate that growth inhibitory properties cannot be correlated with the mere presence of D-amino-acids in peptide linkage. At the outset of the present investigation it was considered likely that, as an additional requirement, the D-amino-acid should be flanked by a suitable pair of amino-acids; it was supposed that when this requirement was met optically selective antibiotic action might appear. A tripeptide possessing the D-amino-acid present in gramicidin S, flanked by the same two amino-acids as in the natural antibiotic peptide has, however, failed to show selective toxicity.

All the available evidence now supports the view expressed by Work (1948) that peptide antibiotics containing D-amino-acids are active, not because they have this character in common, but rather by virtue of the individual structure of the peptide.

In order to eliminate as far as possible the complicating factors of molecular size, and number and type of polar groups per molecule, it seemed desirable to extend the present investigation by the syntheses of appropriate pentapeptides containing the complete amino-acid sequence of gramicidin S; the results of this investigation will be reported in a future communication.

SUMMARY

1. Dipeptides and tripeptides containing D-phenylalanine have been synthesized and compared as growth inhibitors with optically isomeric peptides containing L-phenylalanine.

2. The amino-acids in these peptides were arranged in the same sequence as in gramicidin S.

3. Limited antibiotic activity was observed with synthetic peptides, but no difference in activity was found between a tripeptide containing D-phenylalanine and one containing L-phenylalanine.

4. The significance of these results in relation to the origin of antibiotic activity in peptides is discussed.

We are indebted to Dr A. T. Fuller for the biological results summarized in Table 1.

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The Synthesis of Peptides Related to Gramicidin S and the Significance of Optical Configuration in Antibiotic Peptides

2. PENTAPEPTIDES

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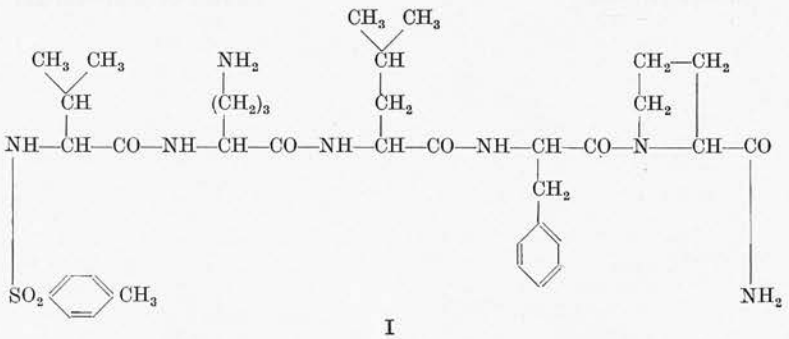
In Part I (Harris & Work, 1950) we reported that esters of both the tripeptides L-leucyl-L-phenylalanyl-L-proline and L-leucyl-D-phenylalanyl-L-proline synthesized by us showed some antibacterial action, but that they were very much less active than the natural antibiotic, gramicidin S, upon which they were modelled. Since there was no clear-cut distinction between the activities of the two optically isomeric peptides, it was decided to extend the peptide chain and to synthesize a pentapeptide containing all the amino-acid constituents of gramicidin S in their proper sequence and optical form, L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl-(II). For comparison with this peptide we synthesized the optically isomeric pentapeptide containing L-phenylalanine in place of D-phenylalanine. By comparison of the antibacterial activity of these two peptides we hoped to reveal the part played by D-phenylalanine in the production of antibiotic action.

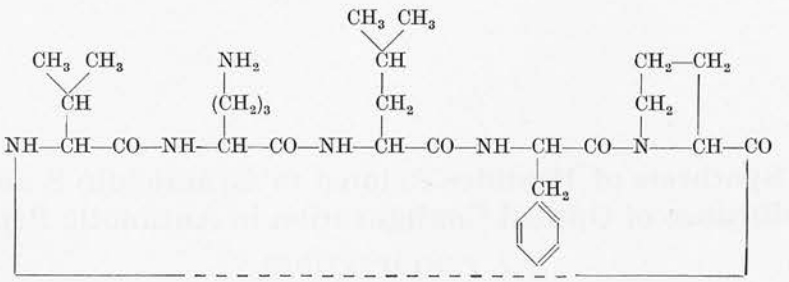
Our two isomeric open-chain pentapeptides, although comparable one with the other, are not quite comparable with gramicidin S since they possess, in addition to the free δ-amino group of the ornithine residue, a free carboxyl and a free α-amino group, both of which are absent from the natural antibiotic. The absence of reactive end groups in gramicidin S is most satisfactorily explained by the assumption of a cyclic structure. We scarcely hoped to achieve controlled cyclization of so complex a molecule as our synthetic 'D' pentapeptide and therefore approached the problem by synthesis of an acylpentapeptide amide, *α-(p-toluenesulphonyl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline-amide*. This compound (I) was comparable to gramicidin S (II) in that it possessed the correct amino-acid sequence, the correct optical form and the desired absence of reactive end groups.

For comparison with the acylpentapeptide possessing a free δ-NH_2 on the ornithine residue, we intended at first to prepare the corresponding pentapeptide with the α-NH_2 of valine free and the δ-NH_2 of ornithine acylated, but, as explained below, this last project was abandoned in view of unexpected experimental difficulties and because of the limited biological activity of our first acylpentapeptide amide.

The choice of the sequence valylornithylleucylphenylalanylprolyl was dictated by experimental rather than theoretical considerations. Since gramicidin S is believed to be cyclic, any one of the five constituent amino-acids can be terminal in an open-chain analogue. Preliminary experiments indicated that some of the possible intermediates would be easier to synthesize and purify than others, and the sequence finally chosen was that which seemed to



I



II

offer the best chance of complete purification and characterization of the intermediate and final products.

Synthetic procedures

The tripeptide esters, L-leucyl-D-phenylalanyl-L-proline methyl ester and L-leucyl-L-phenylalanyl-L-proline ethyl ester were already available from an earlier investigation (Harris & Work, 1950). For preliminary experiments on the lengthening of the peptide chain we used the readily obtainable DL-ornithine rather than the less accessible L-ornithine. *δ-Carbobenzyloxy-DL-ornithine* was prepared initially by the method developed by Neuberger & Sanger (1943) for δ-carbobenzyloxylysine. The yield (49 %) was disappointing. *αδ-Dicarbobenzyloxy-DL-ornithine* was obtained as a by-product. After the completion of these experiments, Synge (1948) also reported a low yield (40 %). In later experiments, using L-ornithine, we found that an excess of alkali raised the yield to 70 %; it is thus apparent that the theoretical quantity of alkali is insufficient to ensure the completion of the reaction between benzyl chloroformate and the copper complex of ornithine. δ-Carbobenzyloxy-L-ornithine methyl ester hydrochloride was prepared by the first method of Synge (1948). The pure ester hydrochloride melted at 141° (uncorr.) $[\alpha]_D^{19°} + 15{\cdot}6°$ in methanol (*c*, 3·0); Synge (1948) reported melting point 132–134°, $[\alpha]_D^{19°} + 14$–$15°$ in methanol (*c*, 4·0). Carbobenzyloxyornithine was not coupled directly to the isomeric tripeptide esters, but was first combined with valine. L-Valine methyl ester hydrochloride prepared in this laboratory by the method of Fischer (1901) melted at 170°, whereas Synge (1948) reported melting point 146–149°.

The synthesis of α-(carbobenzyloxyvalyl)-δ-carbobenzyloxyornithine methyl ester by the azide method proved to be unexpectedly difficult. When carbobenzyloxy-L-valine azide was allowed to react at 0° with δ-carbobenzyloxy-DL-ornithine methyl ester either in ethyl acetate or in ether, the product was an intractable gel. Repeated fractionation of this gel eventually gave a low yield of the desired crystalline product. We suspected at first that our difficulty arose from the use of DL-ornithine instead of L-ornithine, but later, after we had prepared pure L-ornithine by the action of arginase on L-arginine, we were able to show that, in the reaction of carbobenzyloxy-L-valine azide with δ-carbobenzyloxy-L-ornithine methyl ester, the product was equally intractable and the yield equally bad. In the majority of peptide syntheses studied, the azide method has been the preferable method, but in this case the acid chloride method (Synge, 1948) using tosyl valine proved to be much superior.

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide prepared from DL-ornithine was used for coupling with the isomeric tripeptides already synthesized (Harris & Work, 1950). It would have been desirable to use for this synthesis a dipeptide prepared from L-ornithine, but we preferred to reserve our limited supply of L-ornithine for the synthesis of the acylated pentapeptide amide which was to be compared with gramicidin S. Moreover,

we considered it unlikely that the use of DL-ornithine would invalidate our argument with regard to the significance of the optical configuration of phenylalanine. Both *α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxy-DL-ornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester* and *α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxy-DL-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester* were difficult to purify and all attempts at crystallization resulted in the production of stiff gels. After repeated fractional precipitation of the amorphous compounds, correct analyses were obtained for the respective acylated pentapeptide esters and, as a check on their identity, an acid hydrolysate of each was subjected to chromatographic analysis on paper; the presence of the required amino-acids was demonstrated by comparison with an artificial mixture. Although in each case the final product gave almost theoretical analytical figures, we do not regard this as an adequate criterion of purity, and the physical constants quoted for the dicarbobenzyloxypentapeptide esters must be regarded as probable rather than absolute values.

The synthesis of an acylpentapeptide amide with a free δ-amino group on the ornithine residue required the preparation from valine and ornithine of an intermediate acyldipeptide ester with the α-NH_2 of valine and the δ-NH_2 of ornithine protected by groups of different stability. The *p*-toluenesulphonyl ('tosyl') group had a double advantage for this purpose; it was sufficiently stable to be unaffected by the conditions used for removal of a carbobenzyloxy group, and in addition it facilitated crystallization. Tosyl-L-valine was coupled with δ-carbobenzyloxy-L-ornithine methyl ester by the acid chloride method. The product, *α-(tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester*, resembled α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester in having zero rotation.

The conversion of α-(tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester to the related hydrazide required exceptionally drastic conditions, but an excellent yield of hydrazide was eventually obtained by heating the ester under reflux with excess of 100% hydrazine hydrate in anhydrous methanol. The acylated dipeptide hydrazide was converted to the azide which coupled readily with L-leucyl-D-phenylalanyl-L-proline methyl ester to give *α-(tosyl-L-valyl-δ-carbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester*. The crude product resembled the corresponding carbobenzyloxypentapeptide ester in physical properties and tended to set to a stiff gel in all solvents. Crystallization was ultimately effected by extremely slow cooling of a saturated solution, the rate of cooling being controlled by the use of a large Dewar flask (see Experimental section). The final product was in the form of fine needles (Pl. 3*a*). Identity was checked by elementary analysis and by hydrolysis of the compound to its constituent amino-acids, which were then compared chromatographically with an artificial mixture (Pl. 3*b*).

As far as can be ascertained, this is the first recorded synthesis of a crystalline derivative of a pentapeptide built from five different optically pure amino-acids. In order to complete the series of intermediates, a tetrapeptide was synthesized by the coupling of αδ-dicarbobenzyloxy-L-ornithine with L-leucyl-D-phenylalanyl-L-proline methyl ester. The azide method was used for this synthesis.

The tosyl-carbobenzyloxy-pentapeptide ester was converted to the corresponding amide by reaction with ammonia. Selective removal of the carbobenzyloxy group was achieved by catalytic hydrogeneration of the amide. Selective removal by hydrogenation of the ester was also effected. The method of removal of the tosyl group to give the free pentapeptide will be reported in a later communication.

Since δ-carbobenzyloxy-L-ornithine was already available from the above synthesis we sought to couple it with phenylthiocarbonyl-L-valyl chloride. The phenylthiocarbonyl group can be removed under conditions which do not destroy the carbobenzyloxy group (Ehrensvärd, 1947), and in this way we expected to be able to synthesize a pentapeptide possessing a free α-NH_2 on the valine residue and an acyl group on the δ-NH_2 or ornithine. The addition of phenylthiocarbonyl-L-valyl chloride to two equivalents of δ-carbobenzyloxy-L-ornithine methyl ester resulted in rapid spontaneous removal of the protective phenylthiocarbonyl group. Apparently the alkalinity of the ester was sufficient to rupture the unstable thiocarbonyl linkage. *Methyl phenylthiocarbonyl-L-valyl-p-aminobenzoate* was successfully synthesized from phenylthiocarbonyl-L-valyl chloride and methyl *p*-aminobenzoate. In this case the aromatic amino group is much less basic and does not destroy the phenylthiocarbonyl group.

Antibacterial activity of products

The tetrapeptide ester, *L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester* and the isomeric pentapeptide esters α-(L-valyl)-DL-ornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester and α-(L-valyl)-DL-ornithyl-L-leucyl-D-phenylalanyl-L-proline meethyl ester were tested for antibacterial activity, *in vitro*, against *Staphylococcus aureus*, *Streptococcus haemolyticus* and *Escherichia coli*. As can be seen from Table 1, the tetrapeptide ester and the two pentapeptide esters showed only limited activity, and the pentapeptide ester containing D-phenylalanine was not significantly more active than its isomer. The presence of the ester group in these peptides seems to have little influence on activity; thus,

Table 1. *Antibacterial activity of synthetic peptides related to gramicidin* S

(Bacteriostatic concentrations are given in the form $x \times 10^{-y}$ M; the media were as specified in Part 1 (Harris & Work, 1950).)

Substance	*Streptococcus haemolyticus* Blood	*Streptococcus haemolyticus* Broth	*Staphylococcus aureus* Broth	*Escherichia coli* Broth
L-Ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester	—	2×10^{-3}	5×10^{-3}	4×10^{-3}
α-(L-Valyl)-DL-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester	$>1{\cdot}7 \times 10^{-3}$	$1{\cdot}7 \times 10^{-3}$	$>2{\cdot}0 \times 10^{-3}$	$>1{\cdot}7 \times 10^{-2}$
α-(L-Valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline	—	$3{\cdot}5 \times 10^{-3}$	—	—
α-(L-Valyl)-DL-ornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester	—	$3{\cdot}5 \times 10^{-3}$	7×10^{-3}	$>7 \times 10^{-3}$
α-(Tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester	$>1{\cdot}25 \times 10^{-4}$*	$>1{\cdot}25 \times 10^{-4}$*	$>1{\cdot}25 \times 10^{-4}$*	$>1{\cdot}25 \times 10^{-4}$*
α-(Tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline amide	$>1 \times 10^{-4}$*	$>1 \times 10^{-4}$*	$>1 \times 10^{-4}$*	$>1 \times 10^{-4}$*
Gramicidin S	—	5×10^{-6}†	—	—

* Saturated solution. † Calculated on basis of cyclic pentapeptide.

a specimen of α-(L-valyl)-ornithyl-L-leucyl-D-phenylalanyl-L-proline prepared during our study on polymerization of pentapeptides (Harris & Work, unpublished) was found indistinguishable biologically from its ester.

α-(Tosyl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester hydrochloride and α-(tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline amide hydrochloride were sparingly soluble in water and saturated solutions contained 10 and 8 mg./100 ml. respectively; no antibacterial activity was detected at these concentrations, whereas a sample of pure gramicidin S (kindly supplied by Dr L. C. Craig of the Rockefeller Institute, New York) was active at less than one-twentieth of these concentrations.

From the results given in Table 1 it is apparent that open-chain pentapeptides having the gramicidin S sequence of amino-acids possess only limited antibiotic action; in this respect they resemble the optically isomeric tripeptide fragments studied previously (Harris & Work, 1950). Activity does not appear to be directly related to the presence of amino-acids of 'unnatural' D-configuration since there is no appreciable difference in antibacterial potency between a pentapeptide incorporating D-phenylalanine in its structure and the corresponding peptide synthesized from L-phenylalanine.

The open chain pentapeptides differ from gramicidin S in that they possess reactive end groups (amino and carboxyl) in addition to the reactive amino group of the ornithine residue. As is evident from the formulae, synthetic α-(tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline amide (I) bears a close resemblance to the natural antibiotic (II); the only reactive group is the δ-amino of the ornithine residue, but the presence of this polar group in an otherwise 'closed' pentapeptide chain having the correct amino-acid sequence and optical configuration is apparently not the key to activity as was envisaged by Znamenskaya, Agatov & Belozerskiĭ (1948).

On the basis of the chemical and physical evidence so far available, gramicidin S is best formulated as a *cyclic* pentapeptide or decapeptide (Consden, Gorden, Martin & Synge, 1947; Pedersen & Synge, 1948). The experimental results presented in the present paper furnish support for the postulate that the biological aetivity of gramicidin S is intimately related to its *cyclic* structure. Further discussion of this point is reserved for a future communication.

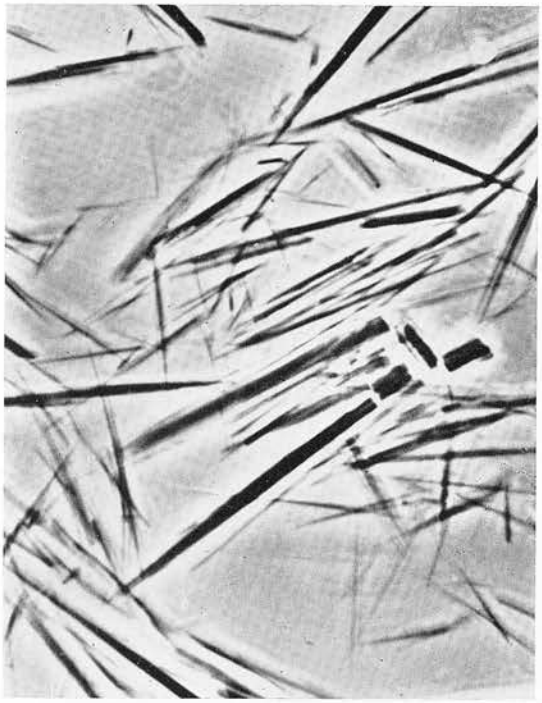
EXPERIMENTAL

Preparative studies

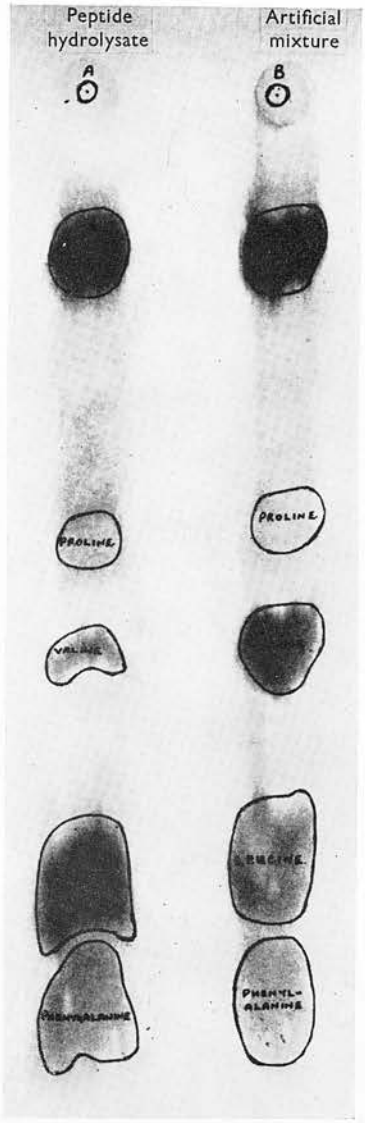
Melting points are given uncorrected; optical rotations were measured in a 4 dm. tube.

*δ-Carbobenzyloxy-*DL*-ornithine.* DL-ornithine was synthesized from acrylonitrile by the acetamidomalonate method of Albertson & Archer (1945), and converted to the δ-carbobenzyloxy derivative by the method described by Neuberger & Sanger (1943) for the preparation of ε-carbobenzyloxylysine. From ornithine monohydrochloride (13 g.), *δ-carbobenzyloxy-*DL*-ornithine* (9·6 g.), m.p. 256°, was obtained in the form of needles. (Found: C, 58·3; H, 6·6; N, 10·7. $C_{13}H_{18}O_4N_2$ requires C, 58·6; H, 6·75; N, 10·5 %.) *αδ-Dicarbobenzyloxy-*DL*-ornithine* (3·2 g.), m.p. 112°, was isolated from the reaction product; this indicated incomplete stability of the Cu complex of ornithine under the conditions used. (Found: C, 63·2; H, 6·4; N, 7·3. $C_{21}H_{24}O_6N_2$ requires C, 63·0; H, 6·0; N, 7·0 %.)

*δ-Carbobenzyloxy-*DL*-ornithine methyl ester.* δ-Carbobenzyloxy-DL-ornithine (12 g.) in anhydrous methanol



(*a*)



(*b*)

(*a*) Photomicrograph by phase contrast of tosyl-L-valyl-δ-carbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester (× 1300).

(*b*) Paper-strip chromatogram of acid hydrolysate of tosyl-L-valyl-δ-carbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester (*A*) run simultaneously on Whatman no. 4 paper with an artificial mixture (*B*) of the component amino-acids; solvent system, 'collidine'-water). Ninhydrin was used for colouring the chromatogram. The yellow colour due to proline did not photograph well. Order of marked spots (from above downwards) ornithine, proline, valine, leucine, phenylalanine.

J. I. HARRIS AND T. S. WORK—THE SYNTHESIS OF PEPTIDES RELATED TO GRAMICIDIN S AND THE SIGNIFICANCE OF OPTICAL CONFIGURATION IN ANTIBIOTIC PEPTIDES. 2. PENTAPEPTIDES

(120 ml.) was saturated with dry HCl gas at room temperature, and left for 24 hr. Concentration *in vacuo* below 30° gave the ester hydrochloride as a syrup. δ-Carbobenzyloxy-DL-ornithine methyl ester hydrochloride was not crystallized. It was converted to the free ester by the usual procedure.

L-Valine methyl ester hydrochloride. L-Valine (20 g.) was converted to the ester hydrochloride by the standard procedure of Fischer (1901). L-Valine methyl ester hydrochloride crystallized from ethanol-ether, and was recrystallized from methanol-ether in the form of lustrous rectangular plates, m.p. 170°. (Found: C, 43·1; H, 8·6; N, 8·0. Calc. for $C_6H_{13}O_2N.HCl$: C, 43·0; 8·4; N, 8·4 %.)

Carbobenzyloxy-L-valine hydrazide. Carbobenzyloxy-L-valine methyl ester (Synge, 1948) (14·0 g., m.p. 56°, prepared from the ester hydrochloride) was dissolved in methanol (100 ml.) and excess hydrazine hydrate (50 % (w/v), 14 ml.) was added. The mixture, left at room temperature for 24 hr., deposited *carbobenzyloxy-L-valine hydrazide* (13·8 g.) which was recrystallized from ethyl acetate-methanol as needles (12 g.), m.p. 179°. (Found: C, 59·1; H, 7·2; N, 16·1. $C_{13}H_{19}O_3N_3$ requires C, 58·9; H, 7·1; N, 15·9 %.)

α-(Carbobenzyloxy-L-valyl-δ-carbobenzyloxyornithine methyl ester. Carbobenzyloxy-L-valine hydrazide (6 g.) in a mixture of glacial acetic acid (50 ml.) and 2N-HCl (100 ml.) was cooled to 0°, and a solution of $NaNO_2$ (1·8 g.) in water (20 ml.) was added dropwise to the stirred solution during 15 min. The acid-insoluble azide was extracted into ice-cold ethyl acetate (150 ml.) and washed successively with ice-cold water and a cold saturated solution of $NaHCO_3$ until the washings were neutral to litmus. The azide solution was dried quickly over Na_2SO_4 and added during 30 min. to a cooled solution of δ-carbobenzyloxy-DL-ornithine methyl ester (7·8 g.) in ethyl acetate (50 ml.). A gelatinous precipitate was formed almost immediately, and after 24 hr. at 0° the whole solution had set to a stiff gel; this was collected by filtration and dried at 80° to give a white amorphous solid (9 g.), m.p. 150–160°. After several precipitations from ethyl acetate a crystalline product was obtained; three recrystallizations from ethanol gave α-(carbobenzyloxy-L-valyl)-δ-carbobenzoxy-ornithine methyl ester as clusters of small needles (1 g.), m.p. 150°, $[\alpha]_D^{17°}$ +11·5° in $CHCl_3$ (*c*, 1·6). (Found: C, 62·6; H, 6·8; N, 8·3. Calc. for $C_{27}H_{35}O_7N_3$: C, 63·1; H, 6·9; N, 8·2 %.) In view of the racemic nature of the ornithine used a definite optical form cannot be assigned to this compound.

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide. To a solution of the methyl ester (0·9 g.) in hot methanol (20 ml.) was added excess 90 % hydrazine hydrate (2 ml.); after 24 hr. at 37°, *α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide* was precipitated as an amorphous gel which was collected and dried at 80° (0·75 g.). (Found: C, 60·6; H, 6·7; N, 13·9. $C_{26}H_{35}O_6N_5$ requires C, 60·8; H, 6·9; N, 13·6 %.)

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester. The hydrazide (0·35 g.) was converted to the azide and extracted into ethyl acetate by the standard procedure already described; this azide was allowed to react at 0° with L-leucyl-L-phenylalanyl-L-proline ethyl ester (0·95 g.) dissolved in anhydrous ethyl acetate (50 ml.). After 24 hr. at 0° and 24 hr. at room temperature, excess tripeptide ester was extracted with N-HCl, and the ethyl acetate layer washed successively with water and saturated aqueous $NaHCO_3$. Removal of solvent under reduced pressure left a pale yellow viscous oil (0·53 g.) which was precipitated as gel, m.p. 124°, from a mixture of ethyl acetate and light petroleum. Several further precipitations from ethyl acetate-light petroleum gave a product (0·4 g.), m.p. 128°, which analysed correctly for *α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester.* (Found: C, 65·0; H, 7·4; N, 9·8. $C_{48}H_{64}O_{10}N_6$ requires C, 65·1; H, 7·3; N, 9·5 %.) The presence of the expected amino-acids in the product was confirmed by paper chromatography of a hydrolysate.

α-(L-Valyl) ornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester. The dicarbobenzyloxy derivative (335 mg.) was hydrogenated in methanol containing 2 equiv. HCl. *L-Valylornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester hydrochloride* was obtained as an extremely hydroscopic semi-solid which was not crystallized.

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. The method used was similar to that described above for the synthesis of the isomeric pentapeptide derivative. α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide (0·35 g.) was converted to the azide and coupled with L-leucyl-D-phenylalanyl-L-proline methyl ester (0·9 g.) in ethyl acetate at 0°. *α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester* was obtained as a colourless, viscous oil which gave a white amorphous solid (400 mg.), m.p. 198–200°, from ethyl acetate-light petroleum. (Found: C, 64·5; H, 7·1; N, 10·0. $C_{47}H_{62}O_{10}N_6$ requires C, 64·8; H, 7·2; N, 9·7 %.) The presence of the expected five amino-acids was confirmed by paper chromatography after acid hydrolysis.

α-(L-Valyl) ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. The dicarbobenzyloxy compound was hydrogenated in methanol containing 2 equiv. HCl, and the pentapeptide ester dihydrochloride isolate as a colourless, hygroscopic syrup (160 mg.) which could not be crystallized.

L-Ornithine. L-Arginine monohydrochloride was isolated from a protein hydrolysate by the flavianic acid method of Cox (1928), and converted into L-ornithine monohydrochloride by the arginase method of Hunter (1939).

Extraction and purification of arginase. A concentrate of arginase was obtained by a modification of the method described by Bach (1946). Fresh ox liver (2·5 kg.) was minced and treated with 5 vol. (10–12 l.) acetone at room temperature. The resulting suspension was filtered, the fibrous residue thoroughly macerated with another 2 vol. (5 l.) of acetone, and filtered again. The fibrous residue was air-dried at room temperature (750 g.). This product (300 g.) was then thoroughly macerated with 0·01N-KOH (500 ml.); a further 1500 ml. KOH solution was added and the suspension stirred for 1 hr. The resulting gelatinous suspension was filtered at 0° and the precipitate washed with 0·002N-KOH. The combined filtrates and washings (3700 ml.) were adjusted to pH 6·8 with HCl and heated in 500 ml. quantities at 54° for 5 min., with gentle shaking. The gelatinous suspension was adjusted to pH 6·1, cooled in ice, centrifuged, and the supernatant liquid was collected (3500 ml.); this liquid was cooled to 0° and the pH readjusted to 6·8. Cold acetone (1·2 vol.; 4200 ml.) was then added with stirring and the mixture left at 0° for 30 min. Adjustment to pH 6 with a few drops of HCl facilitated flocculation of the precipitate, which was centrifuged; the gelatinous protein precipitate was redissolved in the minimum volume of distilled water, and this solution (500 ml.) was treated at 0° with 1·2 vol. (600 ml.) of cold acetone. After keeping at 0° for 1 hr. the pH was adjusted to 6·2 and the resulting enzyme precipitate

collected by centrifugation. The enzyme precipitate dissolved in glass-distilled water (160 ml.) was stored at 0°.

Arginase activity was estimated by the Warburg manometric technique using the arginase-urease method developed by Hunter & Dauphinee (1930). In this way the activity of the arginase solution prepared as above was found to be 300 Hunter units/ml., giving a yield of arginase of approximately 50,000 Hunter units from 1 kg. liver.

From L-arginine monohydrochloride (30 g.), following the procedure of Hunter (1939), L-ornithine monohydrochloride (16·2 g.), $[\alpha]_D^{18°} + 12·02°$ in water (*c*, 4·0), was obtained in 70 % yield.

δ-Carbobenzyloxy-L-ornithine methyl ester. Selective δ-acylation was effected in improved yield by slight modifications of the method used by Synge (1948). L-Ornithine monohydrochloride (5 g.) was converted to the complex copper salt and treated with 1 equiv. 2N-NaOH (15 ml.) at 0°. The resulting deep blue solution was treated with 2N-NaOH (25 ml.; 1·6 equiv.) and benzyl chloroformate (4·5 ml.) added alternately in ten equal portions during 30 min. at 0°. The mixture was stirred at room temperature for another 30 min. and the pale blue precipitate collected by filtration and washed with water and ethanol. After drying, the precipitate was finely powdered, suspended in water (500 ml.) containing HCl (40 ml. 2N), stirred mechanically and decomposed by a stream of H_2S. Precipitated CuS was removed by filtration, and washed thoroughly with hot water.

δ-Carbobenzyloxy-L-ornithine (6·1 g.) was precipitated from the combined filtrate and washings by adding NaOH (40 ml. 2N), and recrystallized from 50 % (v/v) aqueous ethanol, m.p. 254°; yield 5·6 g., 70 % of theoretical.

The methyl ester hydrochloride was obtained in almost quantitative yield by the method described by Synge (1948). Thus, δ-carbobenzyloxy-L-ornithine (5 g.) gave the corresponding methyl ester hydrochloride (5·35 g.) in the form of needles, m.p. 140–141° $[\alpha]_D^{19°} + 15·6°$ in methanol (*c*, 3·0).

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester. Carbobenzyloxy-L-valine hydrazide (1·1 g.) was converted to the azide and allowed to react at 0° with an ethyl acetate solution of δ-carbobenzyloxy-L-ornithine methyl ester (50 % mol. excess; 1·65 g. prepared from 2 g. of the ester hydrochloride). A white gelatinous solid (0·45 g.) which precipitated from the reaction mixture was removed and the acylated dipeptide ester obtained from the filtrate by the procedure already used in the isolation of the corresponding derivative of DL-ornithine. In this way a white solid (1·2 g.) was obtained which crystallized from ethyl acetate as fluffy needles (0·8 g.), m.p. 110–116°. The product had excessive N content and the separation of pure acylated dipeptide ester from impurities proved to be very difficult. Partial purification was achieved by passing a solution of the crude substance (0·8 g.) dissolved in a 20 % (v/v) solution of $CHCl_3$ in benzene (80 ml.) through a column of 'Celite 545' (diatomaceous earth supplied by Johns Manville Co., London, S.W. 1). Crystallization of one of the eluate fractions, from ethyl acetate, gave needles (0·15 g.), m.p. 150°, $[\alpha]_D^{19°} \pm 0°$ in $CHCl_3$ (*c*, 3·0), which analysed correctly as α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester (Synge, 1948). (Found: C, 63·3; H, 6·9; N, 8·4. Calc. for $C_{27}H_{35}O_7N_3$: C, 63·1; H, 6·9; N, 8·2 %.)

αδ-Dicarbobenzyloxy-L-ornithine hydrazide. αδ-Dicarbobenzyloxy-L-ornithine was prepared from L-ornithine monohydrochloride (1 g.) by the method described by Synge (1948). The dicarbobenzyloxy derivative (1·9 g.) which crystallized from $CHCl_3$-light petroleum as fluffy needles, m.p. 114°, was converted through the methyl ester to the hydrazide by the standard procedure. *αδ-Dicarbobenzyloxy-L-ornithine hydrazide* (1·7 g.) was obtained as an amorphous solid, m.p. 126–128°. (Found: C, 60·4; H, 6·4; N, 13·6. $C_{21}H_{26}O_5N_4$ requires C, 60·9; H, 6·3; N, 13·5 %.)

αδ-Dicarbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. αδ-Dicarbobenzyloxy-L-ornithine hydrazide (1 g.) was converted to the azide and coupled with L-leucyl-D-phenylalanyl-L-proline methyl ester (1·4 g.) in ethyl acetate solution at 0°. A small amount of a white gelatinous product (0·1 g.) insoluble in ethyl acetate, formed during the hydrazide-azide conversion, appeared to be αδ-dicarbobenzyloxy-L-ornithine amide (cf. Prelog & Wieland, 1946). The reaction *product* was isolated by the usual procedure as a viscous semi-solid which gave a pale-yellow amorphous solid (1 g.), m.p. 86–88°, on trituration with ether. (Found: C, 64·8; H, 6·7; N, 9·4. $C_{42}H_{53}O_9N_5$ requires C, 65·4; H, 6·9; N, 9·1 %.)

L-Ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. The dicarbobenzyloxy derivative (0·6 g.) was successfully hydrogenated in methanol containing 2 equiv. HCl. Removal of catalyst and concentration of solvent *in vacuo* gave a pale yellow, hygroscopic, amorphous solid (0·4 g.) which did not give satisfactory crystalline material. Provisional identification of the product was achieved by paper chromatography.

Phenylthiocarbonyl-L-valine. Phenylthiocarbonyl chloride was prepared by the method of Rivier (1907). Thiophenol (21 g.) gave phenylthiocarbonyl chloride (24 g.), b.p. 108°/18 mm. Hg, $d_{15°}$ 1·285.

L-Valine methyl ester (2·8 g.) was dissolved in anhydrous ether (40 ml.) and phenylthiocarbonyl chloride (0·5 equiv.; 1·85 g.) in ether (30 ml.) added, with stirring, at room temperature during 5 min. according to the general procedure of Ehrensvärd (1947). After a further 15 min. L-valine methyl ester hydrochloride (2 g.) was collected by filtration; the filtrate was washed with N-HCl and water, dried and concentrated under reduced pressure. *Phenylthiocarbonyl-L-valine methyl ester* crystallized from light petroleum in the form of needles, m.p. 60–62°. Recrystallization from *cyclo*hexanol gave clusters of needles, m.p. 64°; yield 2·3 g. (80 % of theoretical). (Found: C, 58·2; H, 6·4; N, 5·1. $C_{13}H_{17}O_3NS$ requires C, 58·4; H, 6·4; N, 5·2 %.)

The methyl ester (2·1 g.) was heated on a boiling water bath for 20 min. in a 1:1 (v/v) mixture of conc. HCl and glacial acetic acid (20 ml.). Water (40 ml.) was added to the cooled solution and *phenylthiocarbonyl-L-valine* separated as an oil which solidified on standing at 0°. The solid was crushed, washed with water and dried (1·2 g.); concentration of the combined filtrates and washings gave a second crop (0·6 g.), and recrystallization of the combined products from ethyl acetate-ligroin yielded prisms (1·6 g.), m.p. 114°. (Found: C, 56·9; H, 6·13; N, 5·5. $C_{12}H_{15}O_3NS$ requires C, 56·9; H, 6·0; N, 5·5 %.)

Phenylthiocarbonyl-L-valyl-p-aminobenzoic acid methyl ester. Phenylthiocarbonyl-L-valine (0·5 g.) and 1 equiv. (0·44 g.) PCl_5 were suspended in anhydrous ether (10 ml.), and the mixture shaken at room temperature until all the solid material had dissolved. After 1 hr. 2 vol. light petroleum were added and, on cooling the solution below 0°, phenylthiocarbonyl-L-valyl chloride crystallized as needles, (0·35 g.), m.p. 66–68° (sealed tube).

The acid chloride (0·35 g.), dissolved in anhydrous $CHCl_3$ (5 ml.), was added dropwise, with shaking, to an excess of

methyl *p*-aminobenzoate in anhydrous ether (30 ml.), and the mixture left at room temperature overnight. *p*-Aminobenzoic acid methyl ester hydrochloride was collected and the filtrate was kept gently refluxing for 3 hr.; when cool, it was washed successively with N-HCl, saturated aqueous $NaHCO_3$ and water, dried over Na_2SO_4 and concentrated *in vacuo*. The pale yellow *residue* (0·25 g.) was crystallized in the form of white needles (0·15 g.), m.p. 170°. (Found: C, 61·8; H, 5·6; N, 7·5. $C_{20}H_{22}O_4N_2S$ requires C, 62·2; H, 5·7; N, 7·2 %.)

n-p-*Toluenesulphonyl*-L-*valine* (*tosyl*-L-*valine*). To a solution of L-valine (5 g.) in N-NaOH (55 ml.) was added solid *p*-toluenesulphonyl chloride (11 g.); the mixture was stirred vigorously at room temperature for 3 hr. Excess acid chloride was removed by filtration and the filtrate acidified to congo red with dilute HCl. Tosyl-L-valine (Karrer & Veer, 1932), which separated as a white crystalline precipitate, was collected, washed with water and dried. Crystallization from a mixture of ethyl acetate-light petroleum yielded rectangular plates (6·8 g.), m.p. 144°. (Found: C, 53·4; H, 6·1; N, 5·2. Calc. for $C_{12}H_{17}O_4NS$; C, 53·1; H, 6·3; N, 5·2 %.) As there was some danger of racemization in this preparation, a sample of the product was reduced by Na in liquid NH_3 and the valine isolated. The crude valine, as isolated, had a rotation close to that of L-valine.

Tosyl-L-*valyl*-*phenylalanine methyl ester*. Tosyl-L-valine (0·6 g.) and 1 equiv. PCl_5 (0·46 g.) were suspended in anhydrous ether (10 ml.), and the mixture was shaken at room temperature until all the solid material had dissolved. After 1 hr. the ethereal solution was concentrated and 2 vol. dry light petroleum added; tosyl-L-valyl chloride crystallized on cooling the solution below 0° in the form of thin needles (0·4 g.), m.p. 63°.

A solution of this acid chloride (0·4 g.) in ether (20 ml.) was added dropwise with shaking to an ethereal solution of DL-phenylalanine methyl ester (0·7 g., 2 equiv.), and the mixture left at room temperature overnight. DL-Phenylalanine methyl ester hydrochloride (0·4 g.) was removed by filtration, and the filtrate washed free from acid, dried over Na_2SO_4 and concentrated *in vacuo*. The residue (0·4 g.) crystallized from ethyl acetate-light petroleum as white, fluffy needles of *tosyl*-L-*valyl*-*phenylalanine methyl ester* (0·3 g.), m.p. 138–139°. (Found: C, 61·2; H, 6·4; N, 6·8. $C_{22}H_{28}O_5N_2S$ requires C, 61·1; H, 6·5; N, 6·5 %.)

α-(*Tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithine methyl ester*. An ethereal solution of tosyl-L-valyl chloride (0·8 g.) was added to a stirred and cooled solution of δ-carbobenzyloxy-L-ornithine methyl ester (1·7 g., 2 equiv.) in anhydrous ether. A white precipitate formed immediately, and the mixture was left overnight at room temperature to complete the reaction. The thick, white, crystalline precipitate was collected, washed with ether and dried (2·4 g.). Excess δ-carbobenzyloxy-L-ornithine methyl ester was recovered as the hydrochloride by washing the precipitate with water, and the water-insoluble residue (1·48 g.) was recrystallized from acetone-methanol to give the acylated dipetide ester (1·2 g.) in the form of fluffy *needles*, m.p. 187°, $[\alpha]_D^{20°} \pm 0°$ in $CHCl_3$ (*c*, 1·8). (Found: C, 58·8; H, 6·5; N, 7·9. $C_{26}H_{35}O_7N_3S$ requires C, 58·6; H, 6·6; N, 7·9 %.) In large-scale preparations by the same method the yield was 77 %, calculated on the acid chloride taken.

α-(*Tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithine hydrazide*. The conversion of the acylated dipeptide methyl ester to the hydrazide was unusually difficult. After heating under reflux with excess of 90 % aqueous hydrazine hydrate for 2 hr. the ester was recovered almost completely unchanged. The hydrazide was, however, formed by heating the ester (2·5 g.) in anhydrous methanol (50 ml.) under reflux, with 3 equiv. A.R. hydrazine hydrate (100 %) for 6 hr. The hydrazide (2·4 g.) separated as a crystalline solid from the hot solution, and recrystallization from *iso*propanol gave pure *material*, m.p. 226–227°. (Found: C, 56·3; H, 6·6; N, 12·9. $C_{25}H_{35}O_6N_5S$ requires C, 56·3; H, 6·6; N, 13·1 %.)

α-*Tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester*. α-(Tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithine hydrazide (1 g.) was converted to the azide, and a solution of the azide in dry ethyl acetate was added during 15 min. to a cooled solution of L-leucyl-D-phenylalanyl-L-proline methyl ester (1·1 g.; 50 % mol. excess) also in dry ethyl acetate (25 ml.). The solutions were well mixed and left at 0° for 24 hr. A white gelatinous precipitate (0·18 g.), m.p. 200–202°, which had separated at 0° was removed by filtration, and after 24 hr. at room temperature the filtrate deposited a fine amorphous precipitate (0·8 g.), m.p. 204–205°. Excess tripeptide ester was recovered as the hydrochloride by extracting the filtrate with N-HCl. The amorphous product, m.p. 204–205°, was crystallized from methanol by a special technique. The substance was heated under reflux with methanol, filtered hot to remove a small amount of sparingly soluble impurity, and the filtrate left to cool slowly by immersing the securely stoppered containing flask in water at 50° in a large Dewar flask. This procedure ensured a slow steady rate of cooling over a period of 3 days, and in this way α-(*tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester* crystallized as ball-shaped clusters of fine microneedles (3·6 g.), m.p. 200° (Pl. 3*a*). (Found: C, 62·0; H, 7·0; N, 9·3; S, 3·9. $C_{46}H_{62}O_{10}N_6S$ requires C, 62·0; H, 7·0; N, 9·4; S, 3·6 %.)

A second preparation from α-(tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithine hydrazide (1·14 g.) gave the acylated pentapeptide ester (1·4 g.), crystallized by the procedure described above, as aggregates of microneedles, m.p. 201°; identity of the product was confirmed by paper chromatography of a hydrolysate (Pl. 3*b*).

α-(*Tosyl*-L-*valyl*)-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester monohydrochloride*. α-(Tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester (0·5 g.) was dissolved in methanol (100 ml.) containing 1 equiv. HCl. Hydrogenation was effected in the presence of Pd black and evolution of CO_2 was complete in 90 min. After removal of catalyst the filtrate was concentrated *in vacuo*; the oily residue was dissolved in methanol and crystallized by the cautious addition of ether, yielding *microneedles* (0·42 g.), m.p. 236° (decomp.), $[\alpha]_D^{18°} -94·6°$ in methanol (*c*, 1·0). (Found: C, 57·4; H, 7·36; N, 10·7; Cl, 4·3. $C_{38}H_{56}O_8N_6S$.HCl requires C, 57·5; H, 7·2; N, 10·6; Cl, 4·5 %.)

α-(*Tosyl*-L-*valyl*)-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline amide monohydrochloride*. The diacylpentapeptide methyl ester (0·25 g.) was dissolved in methanol previously saturated with anhydrous NH_3 at 0° (10 ml.), and the solution kept at 37° in a sealed tube for 72 hr. The solvent and excess NH_3 were removed *in vacuo*; the solid residue was redissolved in methanol containing 1 equiv. HCl, and hydrogenated in the presence of Pd black by the usual method. After removal of catalyst, the filtrate was concentrated and adjusted to pH 4; the amide hydrochloride crystallized in the form of fine needles, m.p. 256–260° (decomp.), on the

cautious addition of ether. Recrystallization from methanol-ether gave *microneedles*, m.p. 268–270° (decomp.). (Found: N, 13·0%. $C_{37}H_{55}O_7N_7S.HCl$ requires N, 12·6%.)

SUMMARY

1. Two optically isomeric open-chain pentapeptide esters have been synthesized. These peptides have the same sequence of amino-acid residues as gramicidin S.

2. A crystalline acylpentapeptide amide has been synthesized. This compound is structurally analogous to the natural antibiotic in that its constituent amino-acids are arranged in the same sequence and are of the same optical configuration as those of gramicidin S and that its only reactive group is the δ-NH_2 of the ornithine residue.

3. These and other newly synthesized peptides were tested, *in vitro*, for antibacterial activity.

4. The bearing of the results on the question of the origin of antibiotic activity in gramicidin S is discussed.

We are indebted to Dr A. T. Fuller for the biological tests summarized in Table 1, and to Mr J. Smiles for the photograph of crystalline acylpentapeptide ester.

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A discussion on antibiotic activity of growth factor analogues

(Discussion held 17 June 1948 under the leadership of Sir Paul Fildes, F.R.S.)

Selective toxicity, an essential requirement for successful chemotherapy

By T. S. Work

Chemotherapeutic research is judged, ultimately, by its ability to produce new and successful drugs for the treatment of disease. Success may be fortuitous or it may be the result of an inspired guess into the future. It is my task to-day to examine current theories on metabolite analogues and to assess their value as a pointer towards further progress.

A glance at past progress may help us to assess more fairly the present position. Table 4 gives a short list of notable chemotherapeutic drugs together with their origin and date of introduction into European medicine.

Table 4. Historical progress of chemotherapy

drug	date introduction European medicine	remarks
santonin	before A.D. 300	European folk medicine
quinine	1633	borrowed from Americas
ipecacuanha	1658	borrowed from Americas
chaulmoogra	1854	borrowed from India
atoxyl	1905	Thomas
trypan blue	1906	Mesnil & Nicolle
salvarsan	1912	Ehrlich & Bertheim
stibenyl	1916	Caronia
tryparsamide	1919	Jacobs & Heidelberger
Bayer 205	1920	Bayer, A. G.
plasmoquine	1924	Schulemann *et al.*
atebrin	1933	Mauss & Mietzsch
sulphanilamide	1936	Tréfouël *et al.*
sulphapyridine	1938	May & Baker Ltd.
penicillin	1941	Chain, Florey and others
paludrine	1946	Curd & Rose

It is apparent from this table that shortly before 1900 prevailing ideas must have undergone some fundamental change which resulted in a sudden blossoming of the whole subject. The new idea was the germ theory of disease, and its direct offshoot was the subculture of pathogenic organisms both *in vitro* and *in vivo*.

Despite the accumulation of information for over forty years there has been no marked acceleration of the rate of progress, and the production of a new and successful drug is still a long and difficult problem. Indeed, so much is this true, that a prominent research worker in the field recently remarked that he knew only two methods for the production of new drugs—'the hit-and-miss method, where the starting point was a compound of known activity upon which the organic chemist

The arsenic sensitive group of trypanosomes have a high rate of cyanide-insensitive glucose metabolism and are susceptible to selective destruction by arsenicals. Other trypanosomes differ from this group in that their glucose metabolism is highly sensitive to inhibition by cyanide; they cannot be selectively destroyed in the presence of host cells by organic arsenicals (von Brand, Johnson & Rees 1946; von Brand & Tobie 1948). Animals also possess a cyanide-sensitive respiratory system and can probably oxidize glucose in the presence of inhibitors of glycolysis by an alternative metabolic pathway involving phosphogluconic acid (Dickens 1938).

It seems a reasonable assumption, then, that organic arsenicals are effective in eliminating certain trypanosome infections because the trypanosomes concerned differ metabolically from the host cells in being entirely dependent upon a single mechanism of energy production, glycolysis, which is readily inhibited by arsenoxides. This view is supported by the observations of Marshall (1948). *T. evansi*, an arsenic-sensitive trypanosome, is dependent for energy supply on the metabolism of glucose to pyruvate and is unable to utilize other common metabolites such as succinate, fumarate or amino-acids. In this respect it differs markedly from the host cells. Iodoacetate, a specific inhibitor of SH-enzymes, inhibits respiration of *T. evansi* to the extent of over 90 % at a concentration of M/4000; the same inhibitor at a concentration of M/3000 inhibits *T. cruzi* respiration only to the extent of 13 % (Marshall 1948; von Brand *et al.* 1946). The metabolic pathways obviously differ in the two types of cell.

Detailed study of the mode of action of sulphonamides has indicated, as pointed out by Dr Woods at this meeting, that these drugs also owe their selective toxicity to their ability to inhibit a metabolic reaction (formation of pteroylglutamic acid) which is essential to sulphonamide-sensitive organisms but not so essential to the host.

Penicillin is the most outstanding example of selective toxicity among chemotherapeutic drugs. It is highly toxic to a large group of Gram-positive organisms and practically non-toxic to Gram-negative organisms or to animal cells. Gale & Taylor have pointed out that Gram-positive organisms differ from Gram-negative in that they require a number of preformed amino-acids in their medium. Glutamic acid is synthesized readily by Gram-negative organisms and by animals, but has to be supplied in the medium of penicillin-sensitive Gram-positive organisms. It is transported across the cell wall of these organisms by an energy requiring metabolic reaction which is absent from Gram-negative micro-organisms (Gale & Taylor 1946, 1947; Taylor 1947). A strong case has been made out by Gale that the selective toxicity of penicillin is due to inhibition of this metabolic transport reaction which is probably of no importance to the host cells (Gale & Rodwell 1948).

Intensive study of three widely different types of drug has shown, therefore, that chemotherapeutic success is achieved in each case by inhibition of a metabolic reaction which is of vital importance to the parasite but of no particular importance to its host.

Comparative biochemistry has demonstrated many similarities between the metabolic pathways of lower organisms and animal cells. The glycolytic pathway is closely similar in muscle and some microbial cells and is mediated by the same co-enzymes. The tricarboxylic acid cycle is of importance in animal and in microbial

cells; transamination is common to both; phosphorylative transfer of energy through adenosine triphosphate is common to both, and so on. We should not be surprised, therefore, when metabolite analogues of nicotinic acid, of thiamin, of riboflavin or of pyridoxal fail as chemotherapeutic remedies and cause metabolite deficiency in the host as well as in the invading organism. The anti-metabolite approach to chemotherapy demands a much fuller knowledge of the comparative biochemistry of host and pathogen than we yet possess.

Provided we admit our ignorance there is no harm in guessing at the type of metabolite analogue most likely to be chemotherapeutically useful. Indeed, until more is known of intermediary metabolism, particularly on the biosynthetic side, guesswork is essential to further progress.

In the search for metabolic reactions which are essential to pathogen but inessential to or of no importance in the host, there are two related fields which seem to merit attention, amino-acid metabolism and protein synthesis. I have already remarked on some differences in amino-acid metabolism between Gram-positive micro-organisms and their hosts; this difference might be exploited. At Hampstead we have synthesized some amino-acid analogues, but none of these have shown promise as chemotherapeutic agents (Elliott, Fuller & Harington 1948; Harris & Work unpubl.). Other workers have also explored this field without producing any useful new drug (Lichtenstein & Grossowicz 1947; Waelsch, Owades, Miller & Borek 1946; Harris & Kohn 1941; McIlwain 1941). The failure is not wholly surprising; in addition to selective toxicity a drug must also possess some degree of biological stability. The majority of natural α-amino-acids undergo rapid oxidation in the animal body and their analogues probably undergo the same change. Stability can be conferred upon the amino-acid unit by combining it in peptide linkage with other amino-acids; metabolite analogues of such type offer a vast field for further exploration.

TABLE 5. ANTIBIOTIC PEPTIDES

antibiotic	source
gramicidin-D	*Bacillus brevis*
tyrocidine	*B. brevis*
gramicidin-S	*B. sergiev*
subtilin	*B. subtilis*
aerosporin	*B. aerosporus*
licheniformin	*B. licheniformis*
polymyxin	*B. polymyxa*
bacillin	*B. subtilis*
lycomarasmin	*Fusarium lycopersici*

Two known facts make the peptide field particularly attractive, the peptide nature of several natural antibiotics and the species specificity of protein synthesis.

Table 5 gives a list of some antibiotic peptides extracted from micro-organisms.

The addition of licheniformin to this list has only become possible during recent weeks. Licheniformin is a mixture of several closely related antibiotic peptides (Callow & Work unpubl.). The persistent occurrence of amino-acids of unnatural configuration in antibiotic peptides at first suggested that this might be the key to their antibiotic action. To test this hypothesis we made use of the analytical work

If I have seemed, in the latter half of my contribution to this symposium, to allow myself a generous amount of speculation, it is because, for the present, we lack exact knowledge of intermediary metabolism, knowledge which is a prerequisite for the intelligent application of the Fildes-Woods hypothesis to chemotherapy. If my speculations in the field of peptide chemistry do something to stimulate further study of natural and synthetic peptides they will not have been entirely worthless.

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Constituents of Vitamin E Concentrates from Rice- and Wheat-Germ Oils

THE pioneer work of Evans and his collaborators on the anti-sterility factor (vitamin E) has culminated in the isolation from the unsaponifiable fraction of wheat-germ[1] and cotton-seed[2] oils of three apparently isomeric oily alcohols α-, β- and γ-tocopherol of approximate formula $C_{29}H_{50}O_2$, all of which show high vitamin E activity. The use of the unsaponifiable fraction of rice-germ oil as a source of vitamin E was advocated by Kimm[3], who later[4] prepared from a purified concentrate a β-naphthoate, m.p. 156°, which yielded on hydrolysis a product alleged to have very high vitamin E activity.

By acylation of purified concentrates from the unsaponifiable portion of rice-germ oil with *p*-nitrobenzoyl chloride or β-naphthoyl chloride we obtained a complex mixture of oily and crystalline esters. The crystalline esters on separation and hydrolysis yielded three apparently homogeneous crystalline isomeric alcohols of formula $C_{30}H_{50}O$, (*a*) m.p. 121°–122°, (*b*) m.p. 113°–114° and (*c*) m.p. 119°–120°. The alcohol (*c*) yields a β-naphthoate corresponding in its properties to that of Kimm's active material; but like (*a*) and (*b*) it is devoid of vitamin E activity. Of these alcohols, (*a*) may be polyterpenoid in nature but (*b*) and (*c*) are certainly akin to the sterols, being similar in their properties to the tritisterols obtained by Karrer and Salomon[5] from wheat-germ oil concentrates. In a parallel investigation of wheat-germ oil we isolated in similar fashion β-amyrin and two isomeric alcohols $C_{30}H_{50}O$ of the tritisterol type, (*d*) m.p. 113°–114° and (*e*) m.p. 175°; of these (*d*) was also obtained by Karrer and Salomon. Neither (*d*) nor (*e*) possessed vitamin E activity.

The purified oils remaining after removal of these crystalline alcohols from both rice and wheat concentrates had a high biological activity, and gave, on thermal decomposition, considerable quantities of durohydroquinone $C_{10}H_{14}O_2$, which Fernholz[6] obtained by similar treatment of pure α-tocopherol. On treating the oil from the wheat concentrate with cyanic acid in benzene, a mixture of allophanates was obtained from which the products described by Evans, Emerson and Emerson[1] could be isolated, in addition to a crystalline allophanate, m.p. 70°, which has not yet been examined biologically.

The above purified oil from the rice concentrate deposited on standing a crystalline substance, m.p. 73°, which appears to be an aliphatic mono-unsaturated alcohol containing approximately 20 carbon atoms. After removal of this alcohol, the oil on saturation with cyanic acid in benzene solution gave a complex

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CCLXXVI. STUDIES ON VITAMIN E

I. THE ISOLATION OF SOME CRYSTALLINE ALCOHOLS FROM THE UNSAPONIFIABLE MATTER OF RICE AND WHEAT GERM OILS

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(*Received 1 November 1937*)

The unsaponifiable matter from various cereal germ oils is known to possess vitamin E activity, but the majority of investigators appear to have employed solely wheat germ as starting material in attempts to isolate the antisterility factor.

Chemical investigation of rice germ oil was first recorded by Kimm & Noguchi [1933] who identified melissyl alcohol, ergosterol, dihydrositosterol, γ-sitosterol and a new sitosterol, M.P. 156°, as constituents of the unsaponifiable matter from this oil. Later Kimm [1935, 1, 2] employed rice germ oil in an effort to isolate vitamin E. Following the procedure employed by Evans & Burr [1927] in experiments with wheat germ oil, he claimed to have obtained from the unsaponifiable matter an oil which when tested biologically by the Evans method on rats was active in a single dose of 3 mg. This oil on treatment with β-naphthoyl chloride in pyridine yielded four crystalline naphthoates: (*a*) M.P. 168°, (*b*) M.P. 156°, (*c*) M.P. 158°, and (*d*) M.P. 134°. Of these (*b*) was reported to yield on hydrolysis an alcohol showing full vitamin E activity in a single dose of 0·5 mg. Beyond the fact that these esters had colour reactions similar to those of sterol derivatives and that analysis suggested a formula $C_{29}H_{48}O$ for the parent alcohol of (*b*) no further details were recorded.

Some time later Evans *et al.* [1936] reported the isolation from wheat germ oil of two alcohols α- and β-tocopherol. These substances, themselves oils, were separated from the sterol-free unsaponifiable matter of the wheat germ oil as allophanates. The more active substance, α-tocopherol, characterized as its *p*-nitrophenylurethane and allophanate showed vitamin E activity in a dosage of 3 mg., and analysis indicated an approximate formula $C_{29}H_{50}O_2$ in which one oxygen was present in a hydroxyl group and the other perhaps in an ether linkage.

The relatively low activity of α-tocopherol was surprising in view of the results of Drummond *et al.* [1935], who by applying chromatographic analysis to the unsaponifiable matter of wheat germ oil, had earlier obtained vitamin E-containing oils active in dosages of 0·5–1 mg.

In view of these discrepancies in activity and particularly of the difference of an oxygen atom in the formula of "vitamin E" in rice and wheat, we decided to carry out a series of parallel investigations with rice germ and wheat germ oils with a view to the final isolation of the vitamin and elucidation of its structure. The similar order of activity recorded for the preparations of Kimm and Drummond *et al.* led us, in the first place, to concentrate mainly on the use of

the purification process of the latter authors followed by acylation with various reagents. The results of these earlier experiments, concerning which a preliminary note has already been published [Todd *et al.* 1937], are described in this paper.

A number of crystalline alcohols, including Kimm's alleged active alcohol, were isolated from the oils in this way, but they were found to be uniformly devoid of vitamin E activity when tested on rats by the method of Evans. During the progress of our work Karrer & Salomon [1937], reported the isolation from wheat germ oil of compounds of the same type, to which they gave the names α- and β-tritisterol. These authors separated the tritisterols from the unsaponifiable fraction of the oil by precipitating them from 95% alcoholic solution with digitonin, as amorphous, rather unstable digitonides. We have also employed this method in the cases of both rice and wheat with success, the same substances being obtained as by direct acylation without digitonin precipitation.

The compounds from rice, which we designate for convenience as orysterols, are very similar in their properties to the tritisterols of wheat, and indeed one of them may be identical with α-tritisterol. It is however difficult to be quite certain as to the identity of these substances, and in view of the fact that many supposedly homogeneous sterols have been shown to be mixtures we wish to record that, despite the available evidence, we consider it not impossible that the substances we describe may be shown by future work to be in reality mixtures of closely related compounds. As yet we have not investigated the detailed structure of any of the orysterols or tritisterols.

The properties of the various alcohols are described in the experimental part of the paper. All show similarities to the sterols and are characterized by a remarkable tendency to separate from solution in the form of gels even when highly purified; on standing the gels slowly change to a crystalline mass.

Orysterols from rice germ oil

The initial fractionation of the unsaponifiable matter of rice germ oil, after removal of most of the sterols by freezing out, was carried out by chromatographic analysis on activated aluminium oxide (Merck). The rice chromatograms resemble in their general characteristics those obtained from correspondingly treated wheat oils, but show a greater number of individual coloured bands, evidence that rice oil is a much more complex mixture than wheat oil. Further purification was effected by removal of normal sterols by digitonin precipitation and distillation of the oil in a high vacuum. We have found that similar results are obtained when distillation is omitted; vitamin E activity survives distillation and lower-boiling hydrocarbon impurities can be removed by this procedure. By acylation of the purified oils with *p*-nitrobenzoyl chloride or β-naphthoyl chloride we obtained mixtures of esters which could be fractionated to give apparently homogeneous compounds yielding on hydrolysis three alcohols which we provisionally designate α-, β- and γ-orysterols; particulars of these substances are given in Table I.

Table I

	M.P.	$[\alpha]_D$	*p*-Nitrobenzoate M.P.	β-Naphthoate M.P.
α-Orysterol	121–122°	+49°	187–189°	174–177°
β-Orysterol	113–114°	+51·3°	227–228°	166°
γ-Orysterol	119–120°	+51·9°	233–234°	157°

All these substances are unsaturated and have properties similar to those of the tritisterols of Karrer & Salomon [1937] and all are biologically inactive. They

appear to be isomeric alcohols of formula $C_{30}H_{50}O$, but, owing to the extreme difficulty of determining a molecular formula of this nature with complete accuracy, we consider that this formula must await fuller confirmation before it is finally accepted. β- and γ-orysterols as well as their esters show no definite depression in M.P. when mixed with each other although depression is always observed with α-orysterol. In our opinion, however, they are distinct substances, since the crystalline form of the β-naphthoate is quite distinct in the two cases and we were unable to find any evidence in favour of dimorphism as an explanation of the difference. γ-Orysteryl β-naphthoate appears from its M.P. and crystalline form to be the substance isolated by Kimm [1935, 2], but we are quite unable to confirm his claim that the parent alcohol has vitamin E activity.

Neither β- nor γ-orysterol shows a depression in M.P. when mixed with α-tritisterol (M.P. 113–114°) from wheat. Unfortunately we have had insufficient α-tritisterol as yet to permit of definite identification, but from the properties of α-tritisteryl β-naphthoate it seems at least possible that α-tritisterol and γ-orysterol may be identical. It is hoped in further work to settle this point and to ascertain the structure of the orysterols. These substances represent only a small fraction of the rice germ oil (0·12 %). Work on the vitamin E-containing oils left behind after removal of the orysterols will be reported in a separate communication.

Tritisterols from wheat germ oil

As starting material for experiments on wheat germ oil we used a preparation of the unsaponifiable matter from which most of the sterols had been removed by freezing out. This material was purified by partition between light petroleum and methyl alcohol [Evans *et al.* 1936] followed by chromatographic analysis after the manner of Drummond *et al.* [1935] and Karrer & Salomon [1937]. Vacuum distillation of the purified oils was also employed as in the case of our rice experiments. Acylation with *p*-nitrobenzoyl chloride furnished a mixture of oily and crystalline esters. The latter were fractionated with great difficulty as the available quantity was small, but yielded finally on hydrolysis β-amyrin, α-tritisterol, M.P. 113–114°, and a small amount of a third alcohol, M.P. 174–175°, as well as an oily alcohol giving a nitrobenzoate, M.P. 185°. None of these substances showed biological activity. β-Amyrin was isolated by Drummond *et al.* [1935] from wheat germ oil and α-tritisterol was obtained by Karrer & Solomon [1937]. We did not obtain the β-tritisterol described by the latter authors, but this was probably due to the fact that the pre-treatment of our starting material had removed most of the tritisterols present originally, and that we obtained insufficient amounts to permit of complete separation of the various alcohols. The alcohol of M.P. 174–175° may possibly be identical with the alcohol of M.P. 162–163° obtained in traces by Karrer & Salomon [1937], but there was insufficient material to permit of proper characterization.

In one experiment we applied the digitonide isolation process of Karrer & Salomon to our oils but the only product we could identify with certainty was α-tritisterol.

EXPERIMENTAL

(*a*) *Experiments on rice germ oil*

Starting materials. In early experiments we used the crude oil extracted from rice germ with peroxide-free ether. This material was kindly placed at our disposal by Messrs Hoffmann-La Roche, Basle, to whom we wish to express our

β-Orysterol has M.P. 113–114°. (Found: C, 84·3; H, 12·3%; mol. wt. (Rast), 382. $C_{30}H_{50}O$ requires: C, 84·4; H, 11·8%; mol. wt. 426.) In alcoholic solution it is dextrorotatory, $[\alpha]_D^{24°}+51·3°$, and shows only feeble general ultraviolet absorption. In the Liebermann sterol reaction, the free alcohol and its esters show only a reddish yellow colour which rapidly changes to brown, the solution acquiring a green fluorescence. With digitonin in 96% alcohol in the cold, an amorphous precipitate is formed which dissolves on warming. This digitonide breaks up into its components on boiling with absolute alcohol. Tested on rats β-orysterol had no vitamin E activity in a dosage of 5 mg.

β-Orysteryl β-naphthoate. The alcohol (60 mg.) dissolved in pyridine (5 ml.) was refluxed for 3 hr. with β-naphthoyl chloride (0·4 g.), and the solution, after cooling, poured on ice and acidified with sulphuric acid. The precipitate was collected, dried and extracted with boiling light petroleum (B.P. 40–60°). The extract was freed from acid by shaking with sodium carbonate, dried and evaporated. The residue contained, in addition to the desired ester, a considerable amount of β-naphthoic anhydride, M.P. 133–137°, which however was more soluble in acetone than β-orysteryl β-naphthoate; the latter crystallized from acetone or alcohol in colourless plates. (Found: C, 84·2; H, 9·7%. $C_{30}H_{49}O.C_{11}H_7O$ requires: C, 84·4; H, 9·7%.)

A remarkable property of the ester is its behaviour on heating. In an ordinary melting-point apparatus it sinters slightly at 164° and melts sharply to a cloudy, liquid crystalline mass acquiring on further heating a red colour, which at 191° suddenly disappears with formation of a colourless transparent liquid. In thin layers (under the microscope) the appearance of the liquid crystalline phase is somewhat different; at 166° the melt has a lilac colour and on further heating becomes bluish green and strongly iridescent. In this state it is doubly refractive, the clear melt formed at 191° being isotropic. All these changes are observed in the reverse order on cooling and are common to the β-naphthoates of β- and γ-orysterols and of α-tritisterol (see later).

β-Orysteryl acetate. On heating β-orysterol for 90 min. with acetic anhydride, an acetate, M.P. 104°, was obtained which crystallized with difficulty from methyl alcohol.

Light petroleum extract. The extracted material was separated into a more soluble gelatinous fraction and a sparingly soluble fraction, M.P. 193–205° (consisting of a mixture of α-, β- and possibly also γ-orysterol). The residual oily material from the mother-liquors of these fractions was combined with ester from the chromatogram fraction 3.

α-Orysterol. The gelatinous, readily soluble fraction (1·13 g.) of the above extract was dissolved in a mixture of light petroleum and benzene (9 : 1) and adsorbed on a column of activated aluminium oxide (Merck), the chromatogram being developed with *ca.* 1 l. of the same solvent. The middle section of the column, characterized by strong ultraviolet absorption, was eluted with peroxide-free ether and yielded 0·95 g. of a slightly yellowish semi-crystalline mass. The small, brown, top layer of the column was discarded as were the blue fluorescent lower layer and the washings.

This semi-crystalline product was not homogeneous and yielded on crystallization from alcohol a rather gelatinous ester, M.P. 182–189°, as well as some uncrystallizable oil.

The above product (0·8 g.) was hydrolysed by refluxing for 2 hr. with methyl alcoholic KOH (10%), the solution diluted with water, extracted with ether and the extract dried and evaporated. The residual oil crystallized on standing. Recrystallization was difficult as the substance tended to separate from most

solvents as an oil. Crystallization was effected ultimately by slow concentration of an alcoholic solution at room temperature over phosphorus pentoxide. α-Orysterol was thus obtained as small colourless crystals, M.P. 121–122°, after slight sintering at 118°. (Found: C, 84·7; H, 11·6%; mol. wt. (Rast), 390. $C_{30}H_{50}O$ requires: C, 84·4; H, 11·8%; mol. wt. 426.) α-Orysterol gives with digitonin an amorphous precipitate which is moderately soluble in hot 96% alcohol. It shows only feeble ultraviolet absorption with no characteristic maxima, and in alcoholic solution is dextrorotatory, $[\alpha]_D^{25°}+49°$. In the Liebermann test the substance gives a yellow colour which soon changes to brown, the solution at the same time acquiring a green fluorescence.

With one sample of material the colour in the Liebermann test was at first red and changed rapidly through blue to greenish brown, but this was probably due to an impurity.

α-Orysterol is unsaturated towards bromine or potassium permanganate and in biological tests it was found to be devoid of vitamin E activity in doses up to 5 mg.

The M.P. of α-orysterol is depressed on admixture with β- or γ-orysterol.

The *acetate* was obtained as an oil which has not yet been crystallized.

The small amount of oily material remaining in the crystallization mother-liquors of α-orysterol was also inactive in vitamin E tests (dosages up to 4 mg.). Esterification with *p*-nitrobenzoyl chloride in the usual manner gave an ester, M.P. 190–194°.

α-Orysteryl p-*nitrobenzoate.* Esterification of pure α-orysterol with *p*-nitrobenzoyl chloride in pyridine gave an ester, M.P. 187–189°, which tended to form gels from all solvents tried and was only crystallized from alcohol with great difficulty. (Found: C, 77·2; H, 9·0; N, 2·8%. $C_{30}H_{49}O.C_7H_4O_3N$ requires: C, 77·2; H, 9·3; N, 2·4%.)

α-Orysteryl β-naphthoate. On heating with β-naphthoyl chloride in pyridine, α-orysterol gave a β-naphthoate crystallizing from alcohol or acetone in needles, M.P. 174–177°; an unstable liquid crystalline phase could be obtained by supercooling the melt. (Found: C, 84·1; H, 9·0%. $C_{30}H_{49}O.C_{11}H_7O$ requires: C, 84·8; H, 9·7%.) Mixed with the β-naphthoates of β- or γ-orysterol marked depression in M.P. was observed.

Fraction 3. The oily eluate was *p*-nitrobenzoylated but the ester mixture could not be crystallized. It was combined with the oily nitrobenzoate obtained in the working up of fraction 2 (above), hydrolysed and β-naphthoylated. By direct crystallization of the product a naphthoate, M.P. 149–152°, showing a liquid crystalline phase (till 180°) on heating was obtained. The mother-liquors of this substance were submitted to chromatographic analysis on activated aluminium oxide (Merck) previously treated with phenol to reduce its alkalinity; in this way a naphthoate, M.P. 124–128°, was obtained. Owing to the small amounts, these products were not further investigated.

Isolation method II for β- and γ-orysterols. The starting material in this case was a preparation of the unsaponifiable matter of rice germ oil from which a large amount of crystalline sterol had been removed by crystallization. The oil showed full vitamin E activity in rats in a dosage of 125 mg.

The oil (25 g.) was dissolved in light petroleum (500 ml., B.P. 40–60°), filtered, adsorbed on a column of activated aluminium oxide (Merck) and developed with *ca.* 2 l. of the same solvent. The chromatogram was divided into three portions: (*a*) brown layer at top, (*b*) sand coloured main fraction, showing ultraviolet absorption, (*c*) lower yellowish layer. (*a*) and (*c*) were neglected as was also the non-adsorbed oil (6·2 g.). The main fraction (*b*) was eluted with

ether-methyl alcohol (1 : 4); on concentrating the solution obtained, a quantity of sterol (3·9 g.) separated and was filtered off (M.P. 120–130°). The filtrate at this stage had a volume of 220 ml.; it was divided into two equal portions which were worked up separately as follows:

A. The first portion (≡12·5 g. starting material) was precipitated with digitonin (350 ml. of 1 % solution in 90 % alcohol). Only crystalline digitonides (3 g.) separated. On evaporating the filtered solution and extracting the residue with light petroleum an oil was obtained which distilled entirely at 230–260°/0·2 mm. (3·7 g.). The entire distillate was esterified with β-naphthoyl chloride in pyridine. The crude ester (*ca.* 2 g.) crystallized on trituration with acetone and was pressed thoroughly on a filter to get rid of oil.

Successive crystallizations from ethyl acetate, alcohol and acetone gave *γ-orysteryl β-naphthoate*, M.P. 157°. The mother-liquors from these crystallizations furnished on concentration and further purification *β-orysteryl β-naphthoate*, M.P. 166°.

γ-Orysterol. The above naphthoate, M.P. 157°, hydrolysed by refluxing with 10 % butyl alcoholic KOH, yielded crude γ-orysterol, M.P. 104–106°. Like the β-compound γ-orysterol usually separates initially from solution as a gel which quickly crystallizes; crystallization was effected in the first instance from acetic acid-alcohol, and further recrystallization from methyl alcohol gave a final M.P. 119–120°. (Found: C, 83·7; H, 11·9 %. $C_{30}H_{50}O$ requires: C, 84·4; H, 11·8 %.) γ-Orysterol resembles β-orysterol closely in all its properties and no definite depression in M.P. is observed on mixing the two substances. In vitamin E tests on rats γ-orysterol showed no activity in doses up to 5 mg. In alcoholic solution it has $[\alpha]_D^{24.5°} + 51·9°$.

γ-Orysteryl β-naphthoate. The β-naphthoate (see above) has M.P. 157°, with liquid crystalline phase up to 187°. (Found: C, 84·6; H, 9·3 %; mol. wt. (Rast), 520. $C_{30}H_{49}O.C_{11}H_7O$ requires: C, 84·8; H, 9·7 %; mol. wt. 580.)

It crystallizes in needles and shows no clear depression in M.P. when mixed with β-orysteryl β-naphthoate (plates, M.P. 166°); despite numerous efforts, no evidence that these substances were dimorphous forms of one compound could be obtained.

γ-Orysteryl p-*nitrobenzoate.* Esterification of γ-orysterol with *p*-nitrobenzoyl chloride in pyridine gave an ester crystallizing from acetone in leaflets, M.P. 232–233°. (Found: C, 77·3; H, 9·1; N, 2·8 %. $C_{30}H_{49}O.C_7H_4O_3N$ requires: C, 77·2; H, 9·3; N, 2·4 %.) No depression in M.P. was observed on mixing with β-orysteryl *p*-nitrobenzoate (M.P. 227–228°).

B. The second portion of 110 ml. solution was evaporated to dryness and the residue distilled in a high vacuum. The main bulk of the oil distilled with some decomposition at 210–250°/0·1–0·2 mm.

The distillate (5·9 g.) was dissolved in hot 96 % alcohol (100 ml.) and 1 % digitonin solution (300 ml.) added, following the procedure used by Karrer & Salomon [1937] for the tritisterols. The crystalline sterol digitonides were filtered off from the hot mixture and the filtrate on cooling deposited a mass of amorphous digitonides which was collected and washed with absolute alcohol. By repeated concentration of the filtrate and addition of further amounts of digitonin (in all a further 5·7 g.) all the orysterols were precipitated.

The non-precipitable fraction, which we later found to contain all the vitamin E activity, was a brownish waxy mass which could not be crystallized, nor could the oil obtained from it on β-naphthoylation, despite repeated efforts at fractional adsorption and precipitation.

The above amorphous digitonides (8·1 g.) were decomposed by dissolving in

warm pyridine (30 ml.) and precipitating the digitonin with peroxide-free ether (350 ml.) [cf. Schoenheimer & Dam, 1933].

The digitonin-free solution was evaporated and the non-crystalline residue esterified with β-naphthoyl chloride in pyridine. From the mixture of crystalline esters produced β- and γ-orysteryl β-naphthoates could be separated as well as traces of a higher-melting ester (M.P. *ca.* 168°) which may have been largely α-orysteryl β-naphthoate.

Isolation method III. By using methods I and II described above, but omitting the distillation of the concentrates, the same substances were isolated.

(*b*) *Experiments on wheat germ oil*

As starting material for experiments on wheat germ oil we employed a preparation of the unsaponifiable matter from which most of the sterols had been removed by freezing out. This oil, which was kindly supplied by Messrs Glaxo Laboratories, Ltd., to whom we express our thanks, showed full vitamin E activity when tested on rats in a dosage of 125 mg.

The oil (125 g.) was dissolved in light petroleum (1 l., B.P. 100–120°), washed with 92% methyl alcohol and then partitioned between light petroleum and absolute methyl alcohol as described by Evans *et al.* [1936]. The methyl alcohol fraction was concentrated and freed from sterols as far as possible by allowing to stand at 0° for several hours and filtering off the precipitated solid. The resulting oil (35 g.), after removal of methyl alcohol, was distilled in a high vacuum (*a*) B.P. 160–210°; pale yellow oil (10 g.) and (*b*) B.P. 210–255°; orange viscous oil (19 g.). Fraction (*a*) consisted mainly of hydrocarbons and was neglected.

Fraction (*b*) (19 g.) was dissolved in 90% alcohol (1 l.) and the sterols giving crystalline digitonides (*ca.* 8 g.) removed by precipitation with 1% digitonin solution. The solution was evaporated, and the residual oil, freed from digitonin, dissolved in light petroleum (B.P. 40–60°) and adsorbed on a column of activated aluminium oxide (Merck). The chromatogram was developed with the same solvent, and when complete was similar in appearance to the analogous chromatograms of Drummond *et al.* [1935] and Karrer & Salomon [1937]. It was divided into four sections as follows and eluted with a mixture of benzene-acetone-methyl alcohol (8 : 1 : 1) this being found the most effective solvent for elution.

Fraction	Appearance	Eluate
1	Greenish yellow	Dark brown oil (1·2 g.)
2	Sandy	Pale yellow oil (3·0 g.)
3	Yellow	Pale yellow oil (4·2 g.)
4	Colourless	Colourless oil (3·5 g.)
Non-adsorbed	Colourless (3·1 g.)	—

The oil from fraction 2 was found to possess vitamin E activity in a dosage of 25 mg. For further work fractions 2 and 3 were combined.

Isolation method I. The above oil from the combined fractions 2 and 3 (4 g.) was esterified by refluxing (2–3 hr.) with *p*-nitrobenzoyl chloride in pyridine solution and the mixture was poured on ice and acidified with sulphuric acid. The precipitate was collected, dried, extracted with boiling light petroleum and the extract shaken with sodium hydroxide to remove acidic material. After drying, the light petroleum solution was concentrated to *ca.* 50 ml. vol.; on standing a quantity of crystalline material separated, and further concentration yielded a second crop. The combined crystalline fractions were recrystallized from acetone (fraction A). The oil left on evaporating the light petroleum mother-liquor was dissolved in a mixture of equal volumes of alcohol and acetone

(*ca.* 20 ml.). On standing, a crystalline ester separated and was recrystallized from alcohol (fraction B). A further quantity of this ester was obtained by dissolving the oily part in light petroleum and adsorbing on a column of activated aluminium oxide (Merck) previously treated with phenol to reduce alkalinity. The chromatogram was developed with light petroleum containing 2–3% of benzene; apart from a very small colourless fraction (eluate 0·1 g.) at the base and a narrow brown ring at the top (eluate 0·3 g.), the column consisted of two main fractions when viewed in ultraviolet light: (1) an upper colourless layer (eluate 1·3 g.) and (2) a lower highly absorbent layer (eluate 1·0 g.). A quantity of non-adsorbed material passing through the column was discarded.

The eluate from (1), on dissolving in alcohol and allowing to stand, gave a further quantity of fraction B, while the eluate from (3) left for a time in alcohol-acetone solution deposited a small quantity of a third crystalline ester (fraction C). The main bulk of oily esters remaining after removal of these three fractions could not be crystallized.

Fraction A. The substance (140 mg.) crystallized from acetone in colourless needles, M.P. 234–235°. (Found: C, 77·2; H, 9·3; N, 2·7%. $C_{30}H_{49}O.C_7H_4O_3N$ requires: C, 77·2; H, 9·2; N, 2·4%.) It was found on hydrolysis that this apparently pure substance is really a mixture.

The ester was hydrolysed by refluxing with methyl alcoholic KOH in a nitrogen atmosphere. The product, which had the tendency to gel formation noted in the case of the orysterols could be separated by fractional crystallization from methyl alcohol into two components. The less soluble substance formed colourless needles, M.P. 174–175° (3 mg. from 60 mg. ester). On account of the small quantity available it was not possible to characterize the substance more closely or to purify it further. (Found: C, 83·0; H, 11·7%. $C_{30}H_{50}O$ requires: C, 84·4; H, 11·8%.) It showed no vitamin E activity in doses of 3 mg. The Liebermann colour test was similar to that of α-tritisterol.

The more soluble main fraction gave finally a crystalline alcohol, M.P. 113–114°, having the properties recorded for α-tritisterol. (Found: C, 83·7; H, 11·7%. $C_{30}H_{50}O$ requires: C, 84·4; H, 11·8%.) The acetate had M.P. 105–106° (Karrer & Salomon give M.P. 107–108°) and the β-naphthoate formed needles, M.P. 158–159° indistinguishable from those of γ-orysteryl β-naphthoate. Mixed with the latter compound or with β-orysteryl β-naphthoate no depression in M.P. was observed. The Liebermann test gave the same coloration as with the orysterols. No vitamin E activity was shown in doses up to 5 mg. α-Tritisteryl β-naphthoate has also been obtained by Drummond & Hoover [1937] from a wheat germ oil concentrate, although they failed to crystallize the parent alcohol, which they reported as being biologically inactive. They assume the identity of their product with the β-naphthoate of Kimm's active alcohol from rice.

Fraction B. The ester crystallized from alcohol had M.P. 213–215°. (Found: C, 77·1; H, 9·0; N, 2·8%. $C_{30}H_{49}O.C_7H_4O_3N$ requires: C, 77·2; H, 9·3; N, 2·4%.)

This ester appeared not to be homogeneous, as hydrolysis afforded a small amount of a gelatinous alcohol in addition to a main product which, recrystallized first from alcohol and then from light petroleum, had M.P. 197°. (Found: C, 83·7; H, 11·6%; mol. wt. 426. $C_{30}H_{50}$ requires: C, 84·4; H, 11·8%; mol. wt. 426.) That this substance was *β-amyrin* was confirmed by a mixed M.P. with an authentic specimen (M.P. 199°). It yielded an acetate, M.P. 236–237°, undepressed by admixture with authentic β-amyrin acetate (kindly supplied by Dr F. S. Spring to whom we express our thanks).

Fraction C. By recrystallization from acetone an ester, M.P. 185°, was obtained. (Found: C, 77·6; H, 9·2%. $C_{30}H_{49}O.C_7H_4O_3N$ requires: C, 77·2;

H, 9·3%.) Hydrolysis of the ester gave an oily alcohol which had no vitamin E activity in doses of 5 mg.

Isolation method II. The remaining oil from chromatogram fractions 2 and 3 was dissolved in 96% alcohol and the tritisterols precipitated with digitonin according to Karrer & Salomon [1937]. After decomposing the digitonides the sticky mass of alcohols was esterified with β-naphthoyl chloride in pyridine solution. By repeated fractionation of the crystalline ester, *α-tritisteryl β-naphthoate* was obtained as colourless needles, M.P. 158–159°, showing the liquid crystalline phase to 186° noted for γ-orysteryl β-naphthoate. (Found: C, 84·0; H, 9·3%. $C_{30}H_{49}O.C_{11}H_7O$ requires: C, 84·8; H, 9·7%).

From the mother-liquors various fractions were isolated with M.P. ranging from 134 to 160° but no other sharp-melting product could be obtained.

SUMMARY

1. Methods are described for the isolation of three apparently homogeneous alcohols: α-orysterol, M.P. 121–122°; β-orysterol, M.P. 113–114°; γ-orysterol, M.P. 119–120°, from the unsaponifiable matter of rice germ oil. These substances appear to be isomeric and to have an approximate formula $C_{30}H_{50}O$.

2. By similar methods β-amyrin, α-tritisterol, M.P. 113–114°, and a third alcohol, M.P. 174–175°, were obtained from wheat germ oil.

3. All the substances isolated were devoid of vitamin E activity, the activity remaining in the uncrystallizable fractions of the oils.

4. The supposed "vitamin E" of Kimm [1935, 2] corresponds in its properties to γ-orysterol, which is quite inactive.

Our thanks are due to Miss A. M. Copping of this Institute, Prof. E. V. Demole (Messrs Hoffmann-La Roche), Mr A. L. Bacharach (Glaxo Laboratories, Ltd.), Miss M. M. O. Barrie (British Drug Houses, Ltd.), for valuable assistance in biological testing; also to Messrs Hoffmann-La Roche and Glaxo Laboratories, Ltd., for generous gifts of material.

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CCLXXVII. STUDIES ON VITAMIN E
II. THE ISOLATION OF β-TOCOPHEROL FROM WHEAT GERM OIL

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The isolation from wheat germ oil of two isomeric oily alcohols α- and β-tocopherol both showing vitamin E activity has been reported by Evans *et al.* [1936]. These workers purified the unsaponifiable matter of their oil by partition between pentane and methyl alcohol, followed by removal of sterols by freezing out. The concentrated oil thus obtained, active in a dose of 10 mg. on rats, gave on saturation with cyanic acid in benzene solution a mixture of allophanates from which three crystalline esters were separated (*a*) M.P. 250°, (*b*) M.P. 158–160° and (*c*) M.P. 135–138°. The ester, M.P. 250°, was thought to be β-amyrin allophanate, while (*b*)—the main product—gave on hydrolysis an oil, α-tocopherol, showing vitamin E activity in a dosage of 3 mg. and (*c*) yielded a somewhat less active oil, β-tocopherol, on similar treatment.

The tocopherols appeared to be isomeric monohydric alcohols of approximate formula $C_{29}H_{50}O_2$, the second oxygen atom being presumably present in an ether linkage. No yields were given.

Our early experiments on the isolation of vitamin E (see preceding paper) from rice and wheat, having convinced us of the inaccuracy of Kimm's [1935] results and of the impracticability of isolating the vitamin by *p*-nitrobenzoylation or naphthoylation of concentrates, we turned our attention to allophanation in the hope that the results of Evans *et al.* [1936] might be confirmed. At first we endeavoured to repeat the procedure described by these authors, but failed to obtain any definitely crystalline product other than the allophanate M.P. 256–257° (Evans *et al.* give M.P. 250°) derived from an alcohol $C_{30}H_{50}O$. This failure we ascribe to the fact that we were quite unable to obtain, by their method, concentrates from wheat germ oil having anything like the activity they reported. The purified oil of the American authors was active in a dosage of 10 mg.; our similarly treated concentrates were never active in a dosage less than 30 mg. It was therefore clear that further purification would be necessary before application of the allophanate isolation method.

We were able by hydrolysis of the oily esters left after removal of the tritisteryl *p*-nitrobenzoates from distilled wheat germ oil concentrates (see preceding paper) to obtain oils from which poor yields of β-tocopheryl allophanate and an allophanate M.P. 158–159° (apparently not identical with α-tocopheryl allophanate) could be obtained on treatment with cyanic acid. Similar poor yields were obtained by Drummond & Hoover [1937] from their wheat germ oil concentrates. We have since worked out a more satisfactory purification process for the unsaponifiable matter of wheat germ oil, which yields regularly oils showing vitamin E activity in a dosage of about 15 mg. Details of this process are given in the experimental section; it involves partition, chromatographic analysis and

precipitation of tritisterols with digitonin. The oils obtained have the property of reducing ammoniacal methyl alcoholic silver nitrate in the cold with formation of a silver mirror. This property may indeed be used during the later stages of the purification process to determine in which fractions the vitamin occurs, since it is always associated with vitamin E in our experience. On saturating benzene solutions of the active oils with cyanic acid a complex mixture of allophanates is obtained, from which products similar to those described by Evans *et al.* can be isolated.

For the isolation of β-tocopheryl allophanate and the allophanate of M.P. 256–257° simple fractionation with solvents is very unsatisfactory, being attended by considerable loss of material. The isolation of the crystalline allophanates is fairly readily effected, however, by taking advantage of the fact that they are much more strongly adsorbed on activated aluminium oxide (Merck) than the other constituents of the mixture. Elution of the adsorbates with a mixture of benzene, acetone and methyl alcohol, gives a somewhat sticky product from which the crystalline allophanate, M.P. 256–257°, can readily be separated by reason of its very sparing solubility in acetone. Crystallization of the more soluble portion from methyl alcohol yields a trace of an allophanate, M.P. 158–160°, corresponding in M.P. to the α-tocopheryl allophanate of Evans *et al.* [1936] and a very much larger amount of β-tocopheryl allophanate, M.P. 138–139°. Further crystallization of the latter substance yields a product, M.P. 143·5–144·5°, crystallizing in beautiful colourless laths (Fig. 1). A remarkable feature

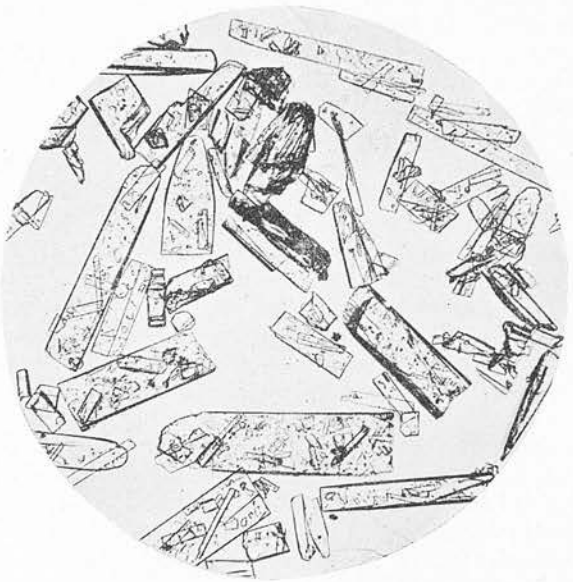

Fig. 1. β-Tocopheryl allophanate ($\times 80$).

of our results is the virtual absence of α-tocopheryl allophanate from our products. From 250 g. of a concentrate of the unsaponifiable matter of wheat germ oil (fully active in a dose of 125 mg.) we obtained at most a few mg. crude "α-tocopheryl allophanate" of doubtful identity, and nearly 1 g. crude β-tocopheryl allophanate. There can be little doubt as to the homogeneity of our β-tocopheryl allophanate, which yields on hydrolysis an alcohol showing full

vitamin E activity in a dosage of 5 mg. It should be emphasized that this is not necessarily the minimum dose which will show activity; owing to the protracted nature of the biological test method the minimum dose for activity will be reported later.

After removal of the crystalline allophanates the residual oil was hydrolysed in a nitrogen atmosphere. The product did not reduce ammoniacal methyl alcoholic silver nitrate solution; moreover, it showed no vitamin E activity in doses of 50 mg. and 100 mg. In view of these facts we are of the opinion that "vitamin E" in our wheat germ oil concentrates consists almost entirely of β-tocopherol.

β-Tocopheryl allophanate appears to have the formula $C_{31}H_{52}O_4N_2$ or $C_{31}H_{50}O_4N_2$, i.e. it is the allophanate of a monohydric alcohol, β-tocopherol, $C_{29}H_{48-50}O_2$, in agreement with the results of Evans *et al.* [1936]. On the available evidence, of course, a C_{30} formula for β-tocopherol cannot be excluded. The allophanate is dextrorotatory in chloroform solution $[\alpha]_{5461}^{21°}+7{\cdot}4°$; we have examined several specimens at different stages of purification and from different isolations and think it unlikely that this rotation is due to impurity.

From certain fractions of the purified oils obtained in the course of our work we obtained by the action of cyanic acid a crystalline allophanate, M.P. 73·5–74·5°, which, from its analytical values, appeared to be the allophanate of a mono-unsaturated alcohol $C_{20}H_{40}O$ containing 3–4 side-methyl groups. Phytol being of common occurrence in nature it seems possible that this substance may be phytyl allophanate.[1]

The allophanate of M.P. 256–257° has not yet been thoroughly investigated. It derives from an alcohol of probable formula $C_{30}H_{50}O$; since β-amyrin allophanate has M.P. 272–273° (Drummond & Hoover [1937] give M.P. 266°) it is unlikely that this alcohol is β-amyrin. We have not obtained the allophanate M.P. 230–232° reported by Drummond & Hoover; this may have been derived from one of the tritisterols.

The chemical nature of β-tocopherol will form the subject of a later communication.

Experimental

Isolation of β-tocopheryl allophanate. As starting material we employed a concentrate of the unsaponifiable matter of wheat germ oil supplied by Messrs Glaxo Laboratories, Ltd., and showing vitamin E activity in a dosage of 125 mg. Throughout our experiments all operations involving heating above room temperature were carried out in a nitrogen atmosphere. The concentrate (250 g.) was dissolved in light petroleum (2 l., B.P. 100–120°) and washed with 92% methyl alcohol. Partition between light petroleum and absolute methyl alcohol was then carried out in the manner described by Evans *et al.* [1936], 5 l. of methyl alcohol (in portions of 500 ml.) being used. The oil obtained from the methyl alcohol fraction in the partition process was redissolved in absolute methyl alcohol (750 ml.) and allowed to stand at 0° during 24 hr. The dark brown semi-crystalline mass (40 g.) which separated was collected, and the filtrate yielded on evaporation a dark brown oil (58 g.). After setting aside 1 g. for various tests, the oil was divided into three equal portions and submitted to chromatographic analysis on activated aluminium oxide (Merck) using a column 35 cm. × 4·5 cm. diam. the solvent used being light petroleum (40–60°). The chromatograms, after developing with light petroleum (*ca.* 5 l.), all had a similar

[1] Since the above was written we have established the identity of this substance with phytyl allophanate (M.P. 78°). Repeated recrystallization raises the M.P. to 77–78°, undepressed on admixture with synthetic phytyl allophanate.

appearance and, viewed in ultraviolet light, showed six distinct zones: (1) narrow, orange layer at top; (2) yellow; (3) sandy, strongly absorbent; (4) large white layer; (5) orange-yellow; (6) blue-fluorescent; the non-adsorbed material was also blue-fluorescent. The column in each case was divided into four sections each eluted separately with benzene-acetone-methyl alcohol (8 : 1 : 1): (A) zone 1 (2·5 cm.), (B) zones 2 and 3 (6 cm.), (C) zones 4 and 5 (14 cm.), (D) zone 7 and washings. The eluates from corresponding fractions were combined giving finally: (A) dark gummy oil (6·7 g.), (B) dark gummy oil (14·7 g.), (C) pale yellow oil (25·5 g.), (D) pale yellow oil (*ca.* 18 g.).

In view of the fact that a small loss was experienced in the above procedure, the aluminium oxide from section B in each column was extracted in a Soxhlet apparatus with acetone-methyl alcohol (1 : 1) and the extract (about 0·1 g. in each case) compared with the main eluate of the corresponding section. The loss due to incomplete elution is probably of no significance in the ordinary way. Sections A, B and C from the chromatogram were examined separately, section D being neglected as far as isolation of vitamin E was concerned.

Section A. This oil had little reducing power and was therefore not further examined for vitamin E. On standing in light petroleum (50 ml., B.P. 40–60°) for several weeks a red carotenoid colouring matter (15 mg.) separated. This substance, as yet unidentified, had M.P. 194° after recrystallization from benzene.

Section B. The oil (14·7 g.) was dissolved in methyl alcohol (100 ml.) and allowed to stand at room temperature during 24 hr.; some crystalline sterol (1·4 g.) was filtered off and the methyl alcoholic solution evaporated. The residual oil was dissolved in 90 % alcohol (250 ml.) and the tritisterols removed by complete precipitation with digitonin (14 g. in all) according to Karrer & Salomon [1937]. The clear solution remaining after this treatment was evaporated to dryness *in vacuo*, taken up in benzene, filtered from digitonin and again evaporated. The residual brown oil (9·5 g.) showed vitamin E activity in a minimum dosage of 15 mg.

Allophanation of tritisterol-free oil from section B. The brown oil (9·0 g.) was dissolved in dry benzene (250 ml.) cooled to 0–5° and saturated with cyanic acid (from 15 g. cyanuric acid heated in a slow stream of CO_2). The benzene solution was kept at 5° for 48 hr., then heated to boiling and filtered from cyamelide, the latter being washed repeatedly with hot benzene. The combined filtrate and washings were evaporated and the residue triturated with cold acetone (100 ml.). A quantity of the allophanate, M.P. 256–257°, was left undissolved and was filtered off. The acetone solution was evaporated and the residual oil dissolved in light petroleum (B.P. 60–80°). On attempting to run this material through a column of activated aluminium oxide the column choked owing to separation of solid material; this difficulty may be overcome by using higher boiling petroleum (B.P. 100–120°), but the following method using the lower boiling solvent worked quite satisfactorily. The solution was shaken with a small quantity of activated aluminium oxide (Merck) (*ca.* 30 g.), filtered and then passed through a column of the same adsorbent (30 × 2 cm. diam.) and developed with light petroleum (B.P. 60–80°). The chromatogram showed three zones in daylight: (*a*) pale brown zone at top (12 cm.), (*b*) orange zone (3 cm.), (*c*) colourless zone (12 cm.). The column was divided into three sections (2, 3 and 4) from the top downwards, 7·5, 7·5 and 12 cm. long respectively, which were separately eluted with benzene-acetone-methyl alcohol (8 : 1 : 1). The alumina used in the preliminary treatment by shaking was also eluted (fraction 1). The weights of the eluted oils were as follows: (1) 3·7 g., (2) 1·65 g., (3) 1·25 g. Fraction (4) gave an

oil which was united with the non-adsorbed material and set aside as containing negligible amounts of active substance.

Fraction (*1*) was dissolved in methyl alcohol (50 ml.) and allowed to stand in the ice-chest. The brownish solid which separated was collected and stirred with cold acetone. A quantity of insoluble allophanate of M.P. about 250° was filtered off, the solution evaporated and the residue dissolved in warm methyl alcohol. On standing, a trace of a product separated having M.P. about 150–154° which was possibly α-tocopheryl allophanate. After filtering this off the mother-liquor deposited a yellowish crystalline mass (550 mg.), M.P. 137–142° (β-tocopheryl allophanate).

Fraction (*2*) gave on similar treatment a further quantity of β-tocopheryl allophanate (130 mg.), M.P. 137–140°, together with a little of the allophanate of M.P. *ca.* 250°.

The non-crystalline residues from sections (1) and (2) were combined, dissolved in light petroleum (B.P. 100–120°) and adsorbed on a column of activated aluminium oxide (30 × 2 cm. diam.). After developing with the same solvent the chromatogram was similar in appearance to the previous one, the upper pale brown zone being divided into two portions: (*a*) 6 cm. yielding 1·35 g. oil, and (*b*) 6 cm. yielding 1·35 g. oil. From (*b*) β-tocopheryl allophanate (250 mg.), M.P. 134–137°, was isolated by crystallization.

Section C. The oil (26 g.) dissolved in light petroleum (B.P. 40–60°) was submitted to chromatographic analysis on a column of activated aluminium oxide (Merck) (40 × 5 cm. diam.). After developing with *ca.* 3 l. of light petroleum the column—which showed seven distinct zones in ultraviolet light—was divided into four sections: (1) yellowish layer (3 cm.) not eluted, (2) absorbent (7 cm.) giving 4·45 g. viscous oil, (3) colourless (17 cm.) giving 12·9 g. limpid yellow oil, (4) orange-yellow and blue fluorescent (7 cm.) giving 6·05 g. yellow oil.

The oil from section (2), on standing in methyl alcoholic solution, deposited some crystalline sterol (0·15 g.); after removing this, tritisterols were removed with digitonin (4 g.) and the remaining oil (3·5 g.) treated with cyanic acid in the usual way and the allophanate mixture fractionated by chromatographic analysis; from the upper layers of the chromatogram β-tocopheryl allophanate (150 mg.), M.P. 137–142°, was obtained together with some allophanate of M.P. about 250°.

The oil (12·9 g.) from section (3) in the above chromatogram was freed from tritisterols with digitonin (5·3 g.) in the usual way and the residual oil treated with cyanic acid as before. From the oily product some allophanate (0·5 g.), M.P. about 250°, was separated by trituration with cold acetone. The acetone solution was evaporated and the residue dissolved in warm methyl alcohol. On cooling some gummy material separated; after filtering the mother-liquor was concentrated to about 50 ml. volume and left in the ice-chest. The oil which separated crystallized from a fresh quantity of methyl alcohol in small crystals (700 mg.), M.P. 70°.

The total yields of the various crude allophanates from 250 g. starting material in the above experiment were thus as follows: allophanate, M.P. *ca.* 250°, *ca.* 1 g.; α-tocopheryl allophanate (?), *ca.* 2 mg.; β-tocopheryl allophanate, *ca.* 1·08 g.; allophanate, M.P. 70°, *ca.* 700 mg.

α-Tocopheryl allophanate (?). The total available amount of this material was so small that it was impossible to identify it properly. It may have been identical with the substance of similar M.P. isolated from the wheat concentrates freed from crystalline tritisterols by acylation (see below). Recrystallized, it had M.P. 158–160°.

β-Tocopheryl allophanate. The combined crude allophanate isolated above had a rather indefinite M.P. 137–140° and separated from methyl alcohol in small needle- or rod-shaped crystals. It still contained a small amount of the allophanate, M.P. 250° (*ca.* 0·1 g.), which could be separated by renewed treatment with acetone. β-Tocopheryl allophanate of M.P. 139–140° is readily obtained by a simple recrystallization from methyl alcohol. Repeated recrystallization from methyl alcohol and acetone yields finally a product of M.P. 143·5–144·5° (Evans *et al.* [1936] give M.P. 138°) crystallizing in beautiful colourless laths (Fig. 1); the purification is, however, accompanied by considerable loss as the substance is rather soluble in methyl alcohol. As the crystallization mother-liquors yield, on working up, only further quantities of the same material, we are of the opinion that the material of M.P. 139–140° is already practically pure.

β-Tocopheryl allophanate analyses in agreement with a formula $C_{31}H_{50}O_4N_2$ or $C_{31}H_{52}O_4N_2$. (Found: C, 72·0; H, 9·9; N, 5·6%. $C_{31}H_{52}O_4N_2$ requires: C, 72·0; H, 10·2; N, 5·4%. $C_{31}H_{50}O_4N_2$ requires: C, 72·3; H, 9·7; N, 5·5%.) In chloroform solution ($c=2·2$; $l=2·2$) it is dextrorotatory, $[\alpha]_{5461}^{21°}+7·4$); various samples were examined and the lowest specific rotation observed was about +6°.

On oxidation with chromic acid (Kuhn-Roth method) β-tocopheryl allophanate gave 3·2 mol. acetic acid. It does not reduce ammoniacal methyl alcoholic silver nitrate solution. Hydrolysed in an atmosphere of nitrogen by heating for 45 min. with 4% methyl alcoholic potassium hydroxide it yields *β-tocopherol* as a slightly yellowish oil, which readily reduces ammoniacal methyl alcoholic silver nitrate. Tested on rats β-tocopherol (from allophanate, M.P. 139–140°) showed full vitamin E activity in a dosage of 5 mg. We are indebted to Dr E. L. Smith (Glaxo Laboratories, Ltd.) for the following spectrographic data: β-tocopherol in alcohol shows a single absorption maximum at 295 mμ ($E_{1\,cm.}^{1\%}=87$).

Allophanate M.P. 250°. Recrystallized from acetone the substance formed small colourless crystals, M.P. 256–257°. (Found: C, 74·7; H, 9·9; N, 5·5%. $C_{32}H_{52}O_3N_2$ requires: C, 74·9; H, 10·2; N, 5·5%.) It has not yet been further examined but does not appear identical with β-amyrin allophanate (M.P. 272–273°).

Allophanate M.P. 70°. Further recrystallization from methyl alcohol gave a product, M.P. 73·5–74·5°, separating in small matted granules. (Found: C, 69·8; H, 10·7%. $C_{22}H_{42}O_3N_2$ requires: C, 69·2; H, 10·9%.) Oxidation with chromic acid (Kuhn-Roth method) gave 2·6 mol. acetic acid (i.e. 3–4 side methyl groups). The substance decolorizes bromine instantaneously.

Oily allophanates. After removal of the crystalline allophanates all the oily fractions were combined and hydrolysed by refluxing in a nitrogen atmosphere with 4% methyl alcoholic KOH. The resulting oil had no reducing action on ammoniacal methyl alcoholic silver nitrate. Moreover, it showed no vitamin E activity in doses of 50 and 100 mg.

Preparation of crystalline allophanates from oils obtained after removal of tritisterols by p-*nitrobenzoylation*

The uncrystallizable oily esters (6·8 g.) left after removal of crystalline *p*-nitrobenzoates in the wheat germ oil isolation method I described in Part I (preceding paper) were hydrolysed by refluxing for 3 hr. with 7% methyl alcoholic KOH. The oil (5·0 g.) was then treated with cyanic acid and the resulting oily allophanate mixture fractionated by adsorption and crystallization in a manner similar to that described above. In this way were isolated (*a*) the allophanate of M.P. *ca.* 250° (100 mg.); (*b*) an allophanate of M.P. 158–159°, corresponding in appearance and M.P. to α-tocopheryl allophanate (*ca.* 8 mg.);

(*c*) β-tocopheryl allophanate, M.P. 138–139° (180 mg.), and (*d*) the allophanate of M.P. 70° (20 mg.).

Allophanate M.P. 158–159°. This material crystallized from methyl alcohol in small granules having an appearance similar to that described by Evans *et al.* for α-tocopheryl allophanate (M.P. 158–160°); the crystals, however, were anisotropic. The analytical values for this material indicate that it is not α-tocopheryl allophanate and is derived from an alcohol of approximate formula $C_{30}H_{50}O$. (Found: C, 75·0; 75·3; H, 10·2, 10·4%. $C_{32}H_{52}O_3N_2$ requires: C, 74·9; H, 10·2%.)

Summary

1. β-Tocopheryl allophanate, M.P. 143·5–144·5°, has been obtained from the unsaponifiable matter of wheat germ oil by an improved method. It is optically active and analysis indicates a formula similar to that suggested by Evans *et al.* The parent alcohol, β-tocopherol, shows an absorption maximum at 295 mμ.

2. The vitamin E activity of our concentrates appears to be due to β-tocopherol, which, when tested on rats, shows full activity in a dose of 5 mg.

3. Although traces of an allophanate of M.P. 158–160° were obtained, it is at least doubtful whether this is the α-tocopheryl allophanate of Evans *et al.* The high-melting allophanate of these authors (M.P. 256–257°) was also obtained, together with an allophanate of M.P. 73·5–74·5° derived from an unsaturated alcohol $C_{20}H_{40}O$.

Our thanks are due to Miss A. M. Copping of this Institute for carrying out the biological tests, and to Messrs Glaxo Laboratories, Ltd. and Messrs Hoffmann-La Roche for gifts of material.

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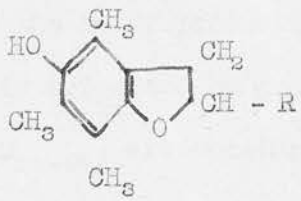
Vitamin E: Structure of β-Tocopherol.

In a recent publication [1] we indicated that β-tocopherol, $C_{28}H_{48}O_2$, might be a coumaran or chroman derivative bearing a long side chain in the heterocyclic nucleus.

With the view of testing this hypothesis, we have synthesized 5-hydroxy-2:4:6:7-tetramethylcoumaran (I), m.p. 124-125°, starting from pseudo-cumoquinol and allyl bromide, and also 5-hydroxy-4:6:7-trimethyl-2-n-heptadecylcoumaran (II), m.p. 95 - 95.5°, isomeric with β-tocopherol, from pseudo-cumoquinone and ethylsodiostearoylacetate followed by partial hydrogenation of the intermediate coumarone.



I R = CH_3

II R = $C_{17}H_{35}$

Both the synthetic coumarans are very similar to β-tocopherol in absorption spectrum and reducing properties.

Substance	Wave-length Max.	Wave-length Min.	mol. Max.	mol. Min.
β-tocopherol	295 mu	260 mu	3577	520
'Heptadecylcoumaran'	297 mu	258 mu	3993	624
'Methylcoumaran'	296 mu	270 mu	3840	1305

When thermal decomposition is attempted, synthetic 5-hydroxy-4:6:7-

trimethyl-2-n-heptadecylcoumaran distils at 370° without charring, giving a mixture from which a trace of a quinol is obtained, m.p. 185-190° (subl.) (cf. thermal decomposition of tocopherols, loc. cit.).

These results together with the surface film measurements of Dr. Danielli, recorded below, give support to the view that β-tocopherol is a coumaran derivative. The detailed configuration of the side chain, and the distribution of CH_3-groups in the ring system remain to be determined. Side methyl estimations of β-tocopherol indicate the presence of six or seven $CH_3(C)$. Two or possibly three of these can be attached to the aromatic nucleus; the remaining three to five can be accounted for by formulating the side chain and the heterocyclic nucleus as a 'dihydrophytyl'-like structure; such a structure would explain also the oily nature of the vitamin and the ready production of quinols on thermal decomposition. This hypothesis is supported by results of oxidation: two oily fatty acids, C_{17-18} and C_{11-12}, are obtained, giving crystalline phenyl-phenacylesters.

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March 10 1938.

(From Nature, 1938, 141, 646)

Reference.

1 Bergel, Todd and Work, J.chem.Soc., 253, (1938).

Nature, 1938, 141, 646.

Both β-tocopherol allophanate and 5-hydroxy-4:6:7-trimethyl-2-*n*-heptadecylcoumarone spread on N/100 hydrochloric acid to give stable films. The limiting area of the first compound is 30 sq. A. and that of the coumarone is 26 sq. A. *p*-Hexadecylcyclohexanol has a limiting area of 30 sq. A. and the lactone of -hydroxystearic acid an area of 29 sq. A. It may therefore be concluded that the first two compounds mentioned are analogous in structure and that in cross-section β-tocopherol cannot have a ring system more than one ring in thickness measured perpendicular to the side chain. The ring system cannot be analogous to a phenanthrene or sterol skeleton.

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March 10 1938.

Reprinted from the Journal of the Chemical Society, 1938.

49. *Studies on Vitamin E. Part III. Observations on the Structure of α- and β-Tocopherol.*

By F. Bergel, A. R. Todd, and T. S. Work.

β-Tocopherol, isolated as its allophanate from wheat germ oil (Part II, *Biochem. J.*, 1937, **31**, 2257) and showing full vitamin E activity in a dosage of 5 mg., is probably identical with the cumotocopherol of John (*Z. physiol. Chem.*, 1937, **250**, 11) and the neotocopherol of Karrer, Salomon, and Fritzsche (*Helv. Chim. Acta*, 1937, **20**, 1422); the name "β-tocopherol" is retained. α-Tocopheryl allophanate is obtained in varying amounts along with β-tocopheryl allophanate from the same sample of wheat germ oil. Pyrolysis of vitamin E concentrates from wheat germ oil gives mixtures of duroquinol and ψ-cumoquinol. Pure β-tocopherol yields on pyrolysis ψ-cumoquinol, accompanied by a small amount of a quinol of higher m. p. Synthetic *cetyl* and *allyl* ethers of duroquinol differ markedly from the tocopherols in absorption spectrum and reducing properties. The possibility that the tocopherols may be chroman or coumaran derivatives is under investigation.

In Part II (*loc. cit.*) we described the isolation of a crystalline optically active allophanate, m. p. 143·5—144·5°, from the unsaponifiable matter of wheat germ oil. This substance yielded on hydrolysis an oil, β-tocopherol, of approximate formula $C_{29}H_{50}O_2$, which showed full vitamin E activity in rats in a dosage of 5 mg. John (*loc. cit.*) and Karrer, Salomon, and Fritzsche (*loc. cit.*) have independently isolated in similar fashion allophanates, m. p. 146° and 143—144°, respectively, which from their physical and chemical properties are almost certainly identical with our compound.

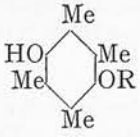
John describes the parent alcohol of his substance as cumotocopherol, and the Swiss workers use the name neotocopherol. We prefer to retain the term β-tocopherol which Evans, Emerson, and Emerson (*J. Biol. Chem.*, 1936, **113**, 319) introduced, since the β-tocopheryl allophanate they described had m. p. 138°, this being the normal m. p. of our crude β-tocopheryl allophanate. The main impurity in this material, m. p. 138°, appears to be a little β-amyrin allophanate, m. p. 273—275°; its removal is a matter of considerable difficulty and is accompanied by a loss of material. The introduction of entirely new names to describe a substance which is almost certainly only a purer specimen of one already in the literature seems to us undesirable. John (*loc. cit.*) states that his tocopherol was active in a minimum dosage of 8 mg., whereas we have had partial activity in dosages as low as 3 mg.; Karrer and his collaborators (*loc. cit.*) give no values for biological activity. This disagreement with regard to biological activity is probably of little significance,

however, as such differences are almost certain to arise where no standardised biological test method is available.

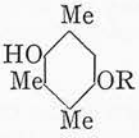
In Part II (*loc. cit.*) we reported that only traces of an allophanate corresponding to the α-tocopheryl allophanate of Evans, Emerson, and Emerson (*loc. cit.*) were obtained. This result we were at first inclined to attribute to a difference between our starting material and that of the American workers, but we have since isolated varying amounts of this compound as well as β-tocopheryl allophanate in different isolations from the same batch of wheat germ oil concentrate. This is the more remarkable as our isolation method has been apparently identical in all cases.

This unaccountable variation in the yield of α-tocopheryl allophanate suggests the possibility that it may be an artefact. The properties and analytical values of our α-tocopheryl allophanate are in agreement with those given by Evans, Emerson, and Emerson (*loc. cit.*) and we confirm their observation of its optical inactivity. The suggestion of Drummond and Hoover (*Biochem. J.*, 1937, **31**, 1852) that β-tocopherol is formed from the α-compound during the isolation process seems unlikely in view of the optical activity of β-tocopheryl allophanate. The total amount of α-tocopheryl allophanate which has been available to us is small, so we have been unable to examine it very closely.

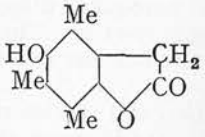
In early experiments (Todd, Bergel, Waldmann, and Work, *Nature*, 1937, **140**, 361) we found, in accordance with Fernholz's results (*J. Amer. Chem. Soc.*, 1937, **59**, 1154), that highly active vitamin E concentrates from rice and wheat germ oils gave duroquinol on heating to about 360°; moreover, concentrates yielding no duroquinol had no biological activity. It was observed, however, that the crude crystalline distillate obtained on pyrolysis generally had a m. p. considerably below 200° and that reasonably pure duroquinol could only be obtained from it after several recrystallisations. Further work on wheat oils showed that this low m. p. was due to the presence in the distillate of varying amounts of a second quinol, m. p. 165—166°, giving on oxidation a steam-volatile quinone, m. p. 28—30°. When slightly impure β-tocopherol was used, this lower-melting quinol was the main product, although some duroquinol was also formed. While these experiments were in progress John (*loc. cit.*) announced that "cumotocopherol" gave ψ-cumoquinol (II; R = H), m. p. 165—170°, on pyrolysis and that it must be an ether of this quinol of possible formula (II; R = $C_{19}H_{37}$) and α-tocopherol correspondingly (I; R = $C_{19}H_{37}$), *i.e.*, that the two compounds were homologues. Our quinol, m. p. 165—166°, we identified by analysis and direct comparison with synthetic ψ-cumoquinol.



(I.)



(II.)



(III.)

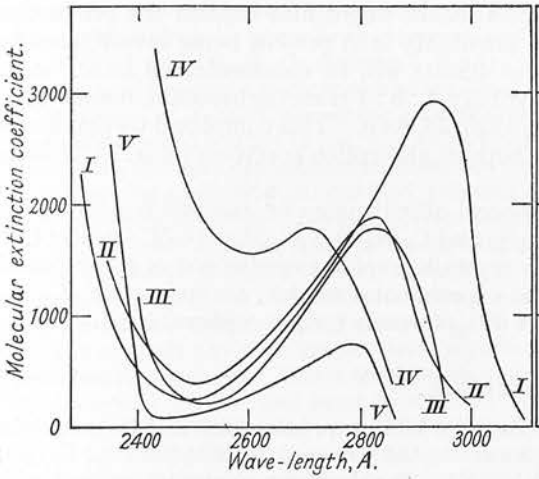
Since the appearance of Fernholz's publication we have synthesised several duroquinol ethers in order to compare their properties with those of the tocopherols. Examination of the absorption spectra of these compounds revealed at once a remarkable difference from the tocopherols. The collected data for the substances examined are shown in Figs. 1 and 2.

Duroquinol and ψ-cumoquinol show single absorption maxima at about 2950 A. In this connection it is worthy of mention that the spectrum given for duroquinol by Karrer, Salomon, and Fritzsche (*loc. cit.*) is incorrect. An absorption curve of the type they describe is only shown by partly oxidised solutions of duroquinol (observation of Dr. H. Waldmann; cf. also John, *loc. cit.*). This oxidation occurs with extreme ease and an accurate spectrum can only be obtained if a freshly prepared duroquinol solution is examined. The mono-ethers of duroquinol also show a maximum, but it occurs at about 2830 A. and is of smaller intensity (mono-acylation of duroquinol causes a similar shift of the maximum); introduction of a second ether grouping as in the dicetyl ether causes a slight shift in the position of this maximum. Esterification of a mono-ether as in *O-benzoylduroquinol allyl ether* causes a further shift of the maximum to about 2710 A.

α-Tocopherol (John, *loc. cit.*) and β-tocopherol show a single absorption maximum at 2950 A. and allophanation of the hydroxyl group (cf. John, *loc. cit.*) causes a shift of the maximum to about 2860 A. accompanied by a marked fall in intensity. On this evidence alone the view that either α- or β-tocopherol is a simple duroquinol ether is highly improbable.

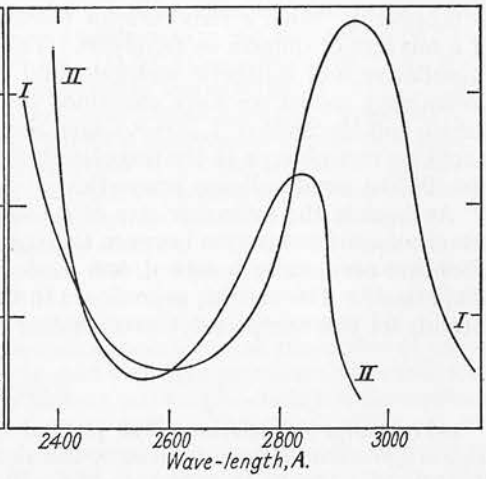
Further evidence pointing in the same direction was obtained by comparing the behaviour of the various compounds towards silver nitrate. Duroquinol, ψ-cumoquinol, and the tocopherols all reduce methyl-alcoholic silver nitrate on warming. This property is not shown by the duroquinol ethers we have prepared, although, of course, the mono-ethers will reduce ammoniacal silver nitrate. Finally, we were unable to isolate either duroquinol or ψ-cumoquinol from the products obtained on heating β-tocopheryl allophanate with hydriodic acid or with hydrochloric acid in acetic acid.

FIG. 1.



I. *Duroquinol in ether.*
II. *Monocetylduroquinol in alcohol.*
III. *Monoallylduroquinol in alcohol.*
IV. *O-Benzoylallylduroquinol in ether.*
V. *Dicetylduroquinol in ether.*

FIG. 2.



I. *β-Tocopherol in alcohol.*
II. *β-Tocopheryl allophanate in alcohol.*

Duroquinol monocetyl ether undergoes pyrolysis much more rapidly than α- or β-tocopherol and at a slightly lower temperature (*ca.* 325°); the crude crystalline distillate of duroquinol has a m. p. above 220° and is already practically pure. The corresponding *dicetyl* ether decomposes at about the same rate as the tocopherols and again yields a very pure product. As mentioned above, we observed the production of both ψ-cumoquinol and duroquinol on pyrolysis of our active materials. We have re-investigated this, using for pyrolysis a specimen of β-tocopherol prepared from a five-times recrystallised sample of the allophanate. Here again we obtained ψ-cumoquinol as the main product, but by washing the crude crystalline distillate, m. p. 160—170°, with ether a small, less soluble fraction of quinol was obtained, m. p. 195—200° with considerable sublimation. The total yield of crystalline material being only about 3% of the weight of tocopherol used, it was impossible for us, with the quantities at our disposal, to identify this material properly. The possibility admittedly exists that our β-tocopheryl allophanate was contaminated with the α-compound, but we consider this highly unlikely. Pyrolysis of a very small amount of α-tocopherol yielded a crude crystalline material of m. p. between 180° and 190°, again suggestive of a mixture of duroquinol with a lower-melting quinol, when contrasted with the behaviour of duroquinol cetyl ether. The evidence is admittedly not conclusive, but we consider it at least possible that both α- and β-tocopherol yield mixtures of ψ-cumoquinol and duroquinol on pyrolysis.

As has been pointed out by Evans, Emerson, and Emerson (*loc. cit.*) α-tocopheryl

allophanate does not behave like an unsaturated compound towards bromine or potassium permanganate; the same is true of β-tocopheryl allophanate. Work on the oxidative degradation of β-tocopherol has been hampered by scarcity of material, but oxidation of the allophanate with alkaline potassium permanganate gave an acid yielding a p-*phenylphenacyl* ester, m. p. 84°. The small amount available precluded complete identification, but it appeared from analysis to be an aliphatic acid containing about 18 carbon atoms. Catalytic hydrogenation of β-tocopherol is difficult to effect, but 4 mols. of hydrogen were taken up slowly on heating. The allophanate also absorbed 4 mols. of hydrogen, but here, curiously enough, 1 mol. was rather easily taken up.

The properties of α- and β-tocopherol so far as they are known indicate that they are in some way related to duroquinol and ψ-cumoquinol. The absorption of 4 mols. of hydrogen by β-tocopherol, coupled with the apparently saturated nature of its allophanate, suggests the possibility that it may be a cyclic ether, *i.e.*, a coumaran or chroman derivative bearing a long side chain in the heterocyclic nucleus. A similar structure would be expected for α-tocopherol. Such a structure for these compounds might also explain the production of a mixture of quinols on pyrolysis. This possibility is at present being investigated by degradative and synthetic methods, and our results will be communicated later. As a preliminary model we have examined 5-hydroxy-4 : 6 : 7-trimethyl*iso*coumaranone (III) (Smith and MacMullen, *J. Amer. Chem. Soc.*, 1936, **58**, 630). This compound we find shows a striking resemblance to the tocopherols in both its absorption spectrum (max. *ca.* 2930 A., min. 2650 A.) and reducing properties.

As regards the molecular size of β-tocopheryl allophanate our analysis figures do not permit of a differentiation between $C_{30}H_{50}O_4N_2$ and $C_{31}H_{52}O_4N_2$. Miss D. M. Crowfoot, to whom we are greatly indebted, has made a crystallographic examination of β-tocopheryl allophanate. Her results, reproduced in the experimental section, are indicative of a C_{30} formula for this compound, corresponding to a C_{28} formula for β-tocopherol itself.

Experimental.

α-Tocopheryl Allophanate.—The purified substance had m. p. 158—159° and its properties were in agreement with those given by Evans, Emerson, and Emerson (*loc. cit.*) (Found : C, 72·3; H, 10·0. Calc. for $C_{31}H_{52}O_4N_2$: C, 72·0; H, 10·2%). In chloroform solution it was optically inactive. α-Tocopherol was obtained from it by hydrolysis as a colourless oil.

β-Amyrin Allophanate from Crude β-Tocopheryl Allophanate.—The crude β-tocopheryl allophanate, m. p. *ca.* 138° (cf. Part II, *loc. cit.*), shows a reddish-violet colour in the Liebermann reaction, whereas the pure allophanate, m. p. 143·5—144·5°, does not. Purification is best effected by boiling methyl-alcoholic solutions with charcoal; the charcoal, then eluted with acetone, yields small amounts of a beautifully crystalline allophanate, m. p. 273—275°, showing the reddish-violet Liebermann reaction characteristic of β-amyrin derivatives. Mixed with a specimen of β-amyrin allophanate (m. p. 272—273°), it produced no depression of the m. p.

Micro-hydrogenations.—β-Tocopherol (1·02 mg.), $C_{29}H_{50}O_2$, absorbed 0·2055 c.c. of hydrogen at N.T.P. (= 3·87 mols. H_2). The solvent was decalin–acetic acid, and the catalyst platinum oxide. No absorption occurred in the cold, but slow regular absorption occurred at 90° and was complete after 8 hours.

β-Tocopheryl allophanate (1·905 mg.), $C_{31}H_{52}O_4N_2$, absorbed 0·3274 c.c. of hydrogen at N.T.P. (= 3·96 mols. H_2), the solvent being acetic acid, and the catalyst platinum oxide. One mol. of hydrogen was absorbed in the cold, and the remainder by heating the mixture to 90°, shaking it until it was cold, and keeping it for 12 hours.

Attempted Acid Fission of β-Tocopherol.—β-Tocopheryl allophanate (100 mg.) was heated in a sealed tube at 200° with hydrochloric–acetic acid (5 c.c. of a mixture of 10 c.c. of glacial acetic acid and 30 c.c. of concentrated hydrochloric acid saturated with hydrogen chloride at 0°) during 18 hours. After a further 2 hours at 220° the tube was cooled, and the dark gummy product extracted with ether. The ethereal solution, on drying and evaporating, gave a dark brown gum which did not crystallise. Oxidation with ferric chloride gave a mixture which had a slight quinone-like odour, but no appreciable steam-volatile product could be detected. The combined material from this experiment was refluxed for 3 hours with hydriodic acid (*d* 1·7), and the product submitted to chromatographic analysis on aluminium oxide (Merck). A small amount of a yellowish oil was washed through the column. The rest of the material, which was

very strongly absorbed, was a brown gum; this did not crystallise, nor did it appear to give a volatile quinone on oxidation.

Oxidation of β-Tocopheryl Allophanate.—The allophanate (145 mg.), dissolved in pure hexane (50 c.c.), was shaken for 12 hours with a solution of potassium permanganate (570 mg. ≡ 19 mols.) in sodium hydroxide solution (75 c.c. of 3%). Much of the permanganate was then unchanged and the concentration of alkali was increased to 6% and shaking continued for a further 24 hours. The hexane layer was separated and gave on evaporation a small amount of a substance, m. p. 140°, possibly unchanged allophanate. The aqueous layer was freed from manganese dioxide and unchanged permanganate by means of sulphur dioxide, and the resulting solution, after acidification with sulphuric acid, was extracted continuously with ether during 48 hours. The extract was shaken with alkali. A small amount of an unidentified neutral oil remained in the ether, and the acidic material was recovered by acidifying the alkaline layer and again extracting it with ether. Evaporation of this ethereal extract gave a thick oil (26 mg.). This oil had no detectable rotation in chloroform solution ($c = 0{\cdot}65\%$; $l = 1$ dm.) and titration against N/20-sodium hydroxide indicated a mol. wt. of about 260, assuming it to be a monobasic acid. The material was esterified with *p*-phenylphenacyl bromide, and the *ester* crystallised from methyl alcohol. After several recrystallisations a product, m. p. 84°, was obtained (Found: C, 80·7; H, 9·4. $C_{33}H_{46}O_3$ requires C, 80·3; H, 9·6%. $C_{33}H_{44}O_3$ requires C, 80·7; H, 9·3%). A portion of the crude acidic material remained unesterified in this experiment.

Pyrolysis of Active Concentrates from Wheat Germ Oil.—The oil (600 mg.), prepared by the method described in Part II (*loc. cit.*) and showing vitamin E activity in a dosage of 15 mg., was heated in a nitrogen atmosphere to about 360° in a bulb tube with a long side arm. From about 300° onwards a yellowish oil distilled, followed at 350—360° by a product which crystallised in colourless needles in the side tube. After about 20 minutes the distillation was complete. The crystalline distillate was freed from oily impurities by washing with a little light petroleum (b. p. 40—60°); it had a rather indefinite m. p. 170—180°. The material was recrystallised from petroleum (b. p. 100—120°) and the crystals (A) separating were washed with low-boiling petroleum, the washings being combined with the crystallisation mother-liquors (B).

The crystals (A) were treated with a little sodium hyposulphite solution, filtered off, and sublimed in a vacuum. The sublimate was again washed with light petroleum, then with a very small amount of ether, and again sublimed. The product (*ca.* 6 mg.), which had the crystalline form of duroquinol, had m. p. 223—224° (Found: C, 72·5; H, 8·6. Calc. for $C_{10}H_{14}O_2$: C, 72·3; H, 8·4%). A mixed m. p. with synthetic duroquinol showed no depression, and oxidation with ferric chloride gave a yellow quinone, m. p. 107—108°, undepressed by duroquinone (m. p. 108—109°).

The combined liquors and washings (B) were evaporated, and the residue washed with sodium hyposulphite solution and taken up in ether. The dried ethereal solution was evaporated, and the residue purified by crystallisation from light petroleum (b. p. 60—80°) and sublimation in a vacuum. The product (2·5 mg.) had m. p. 165—166° (Found: C, 70·7; H, 8·1. Calc. for $C_9H_{12}O_2$: C, 71·1; H, 7·9%). A mixed m. p. with ψ-cumoquinol (m. p. 168—170°) showed no depression and oxidation with ferric chloride gave a yellow quinone, m. p. 28—30° (ψ-cumoquinone has m. p. 30°).

The relative proportions of the two quinols varied in different experiments even with the same sample of oil.

Duroquinol Cetyl Ethers.—A mixture of duroquinol (3 g.), methyl ethyl ketone (80 c.c.), cetyl iodide (6·3 g.), and anhydrous potassium carbonate (2·55 g.) was refluxed in a nitrogen atmosphere during 10 hours. The mixture was now filtered, and the filtrate kept at 0° for a few hours. The crystalline material which separated was dissolved in light petroleum (b. p. 40—60°), and the filtered solution evaporated. The residue, recrystallised from acetone, gave *duroquinol dicetyl ether* in colourless waxy crystals, m. p. 81—84° to a cloudy liquid which became clear at 86—87° [Found: C, 82·0; H, 12·5; *M* (Rast), 550. $C_{42}H_{78}O_2$ requires C, 82·2; H, 12·5%; *M*, 614].

The methyl ethyl ketone filtrate from the dicetyl ether was evaporated to dryness, and the residue freed from remaining traces of the latter compound by dissolution in cold acetone. The acetone solution was now steam-distilled to remove duroquinone, and the distillation residue shaken with light petroleum. A yellow solid (0·85 g.) remained undissolved; it had m. p. 204—205° after recrystallisation from methyl alcohol and was probably diduroquinone (v. Pechmann, *Ber.*, 1889, **22**, 2115). The light petroleum solution was washed with aqueous caustic potash, then with water, dried over sodium sulphate, and evaporated. The residue was taken up in a small amount of acetone, in which *duroquinol monocetyl ether* is readily soluble, filtered, and cooled to − 5°. The crystalline material which separated had m. p. 91—92°, but

still contained traces of iodine (contamination with cetyl iodide). By repeated crystallisation from methyl alcohol and finally from light petroleum (b. p. 60—80°) the ether was obtained in colourless waxy crystals, m. p. 98° (Found: C, 79·4; H, 11·4. $C_{26}H_{40}O_2$ requires C, 80·0; H, 11·7%). On oxidation with chromic acid by the Kuhn–Roth method the substance gave 3·2 mols. of acetic acid. It showed no vitamin E activity when tested biologically in doses up to 50 mg.

At about 325° the monocetyl ether decomposes within a few minutes, giving duroquinol (m. p. crude 221°). The dicetyl ether behaves similarly, but the decomposition occupies about 20 minutes (m. p. of crude duroquinol 225—227°). The yield in each case is *ca.* 20% of the theoretical.

O-*Monobenzoylduroquinol.*—To a solution of duroquinol (5·2 g.) in dry pyridine (26 c.c.) at 0°, benzoyl chloride (3·8 c.c.) was added dropwise with shaking, the whole operation being carried out in an atmosphere of nitrogen. After standing overnight, the mixture was heated on the water-bath for 1 hour, cooled, and poured on a mixture of ice and dilute sulphuric acid. The solid which separated was collected, washed thoroughly first with sodium hydroxide solution, then with water, dried, and boiled with methyl alcohol (80 c.c.); this treatment left much of the product undissolved but removed duroquinone and unchanged duroquinol. After cooling, the suspension was filtered; the filter residue (5·2 g.) had m. p. 204—215°. The product was extracted three times with a mixture of benzene (4 parts) and petroleum (7 parts; b. p. 100—120°), which removed the more soluble dibenzoyl derivative. The residue (4 g.), m. p. 220—221°, gave after two recrystallisations from methyl alcohol colourless prismatic needles of O-*monobenzoylduroquinol*, m. p. 221—223° (Found: C, 75·6; H, 6·4. $C_{17}H_{18}O_3$ requires C, 75·6; H, 6·6%).

O-*Benzoylduroquinol Allyl Ether.*—*O*-Monobenzoylduroquinol (0·6 g.) was added to potassium powder (0·1 g.) in toluene (20 c.c.) and heated, a thick gelatinous mass being formed. When most of the potassium had disappeared, allyl bromide (0·24 c.c.) was added together with more dry toluene (10 c.c.), and the whole refluxed for *ca.* 4 hours; the gelatinous material then disappeared and potassium bromide separated. The liquid was now filtered and evaporated to dryness, yielding a thick oil which crystallised on trituration with light petroleum. Recrystallised from methyl alcohol, O-*benzoylduroquinol allyl ether* formed colourless needles (0·4 g.), m. p. 111—112° (Found: C, 76·9; H, 7·0. $C_{20}H_{22}O_3$ requires C, 77·4; H, 7·1%).

Duroquinol Monoallyl Ether.—The above benzoate was refluxed in a nitrogen atmosphere with methyl-alcoholic potassium hydroxide (5%) during 2 hours. The resulting solution was diluted with water and extracted with ether, and the extract dried and evaporated. The oily residue crystallised from light petroleum (b. p. 40—60°) in colourless needles, m. p. 108° (Found: C, 75·8; H, 8·8. $C_{13}H_{18}O_2$ requires C, 75·7; H, 8·7%). The substance reduced ammoniacal methyl-alcoholic silver nitrate solution but had no action on methyl-alcoholic silver nitrate alone, even on heating. Duroquinol monocetyl ether also may be prepared in an analogous manner *via* the benzoyl derivative; a sample thus prepared had m. p. 102°.

Migration Experiments.—When a solution of *O*-benzoylduroquinol allyl ether in dimethylaniline was refluxed for 6 hours, the allyl group was removed and *O*-monobenzoylduroquinol, m. p. 221—223°, was isolated in nearly quantitative yield. On similar treatment duroquinol monoallyl ether yielded duroquinone (oxidation of initially formed quinol), but duroquinol monocetyl ether was recovered unchanged.

Crystallographic Data.—β-Tocopheryl allophanate crystallises in the monoclinic space group P_2; $a = 13{\cdot}52$ A. (limits, 13·57, 13·45); $b = 5{\cdot}26$ A. (limits 5·27, 5·245); $c \sin \beta = 21{\cdot}35$ (limits 21·40, 21·05); $p = 1{\cdot}092$ (limits 1·097, 1·087). The number of molecules in the unit cell being taken as 2, the minimum molecular weight required by the crystallography is 502 (limits 509, 490). This indicates definitely a C_{30} formula ($C_{30}H_{50}O_4N_2$ requires M, 502).

We are indebted to Mr. F. Boston, Manchester University, for the micro-hydrogenation figures. Our thanks are also due to Messrs. Glaxo Laboratories Ltd. and Messrs. Hoffmann La Roche & Co. for their generous assistance and gifts of material and to Dr. E. L. Smith of the former firm for measuring many absorption spectra. We have to thank Miss. A. M. Copping, who has undertaken the biological testing necessary in these investigations, and the Medical Research Council, whose grant to her has made this possible.

(Note added, February 11th) Emerson, Emerson, Mohammad, and Evans (*J. Biol. Chem.*, 1937, **122**, 99) have now reported that their purified β-tocopheryl allophanate has m. p. 144—146°.

THE LISTER INSTITUTE, LONDON, S.W. 1. [*Received, December 20th*, 1937.]

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Vitamin E Synthesis of α-Tocopherol

THE recent announcement by Karrer, Fritzsche, Ringier and Salomon[1] makes it desirable for us to place on record the fact that we have also synthesized racemic α-tocopherol by a rather simpler method, namely, direct condensation of phytol with ψ-cumoquinol by heating a mixture of these two substances in presence of a little zinc chloride. This synthesis, like that of the Swiss workers, while it confirms the view originally expressed by ourselves[2] and by Fernholz[3] that the tocopherols are chroman or coumaran derivatives, nevertheless fails to distinguish between the two types of structure.

Karrer's arguments[4] in favour of a coumaran structure for the synthetic product rest on an assumption that phytyl bromide will condense with a phenol in exactly the same way as allyl bromide. This assumption seems to us unjustifiable, and it is indeed more probable that condensations of this type would lead to chroman structures when phytol derivatives are used. Recent degradative evidence, although inconclusive, is on the whole more indicative of a chroman structure for the tocopherols[3,5].

On the synthetic side we have found that 6-hydroxychromans, 5-hydroxycoumarans, and α- and β-tocopherol are nearly identical as regards absorption spectrum, reducing properties, and effect of esterification on absorption spectrum.

We are at present engaged on the synthesis of the tocopherols by unequivocal methods, since it seems that only in this way can a final decision be reached as to their structure.

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June 17.

[1] NATURE, **141**, 1057 (1938).
[2] Bergel, Todd and Work, *J. Chem. Soc.*, 253 (1938); Bergel Jacob, Todd and Work, NATURE, **141**, 646 (1938).
[3] Fernholz, *J. Amer. Chem. Soc.*, **60**, 700 (1938).
[4] Karrer, Fritzsche, Ringier and Salomon, *Helv. chim. Acta*, **21**, 520 (1938).
[5] Dietzel, Günther and Emte, *Naturwiss.*, 366 (1938).

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257. *Studies on Vitamin E. Part IV. Synthetic Experiments in the Coumaran and Chroman Series. The Structure of the Tocopherols.*

By F. Bergel, (Miss) A. Jacob, A. R. Todd, and T. S. Work.

Confirmation of the view expressed in Part III of this series that the tocopherols are chroman or coumaran derivatives bearing a long side chain in the heterocyclic nucleus has been sought by model synthetic experiments. ψ-Cumoquinone, condensed with ethyl sodiostearoylacetate, gave as main product 5-*hydroxy*-3-*stearoyl*-4 : 6 : 7-*trimethyl*isocoumaranone from which was prepared 5-*hydroxy*-4 : 6 : 7-*trimethyl*-2-n-*heptadecylcoumaran*. This compound is isomeric with β-tocopherol and resembles it closely in absorption spectrum and reducing properties, but it has no vitamin E activity in doses up to 100 mg. Condensation of ethyl sodiopalmitoylacetate and ψ-cumoquinone proceeded similarly. 5-*Hydroxy*-2 : 4 : 6 : 7-*tetramethylcoumaran*, synthesised by two methods, also resembles the tocopherols closely in properties. Similarly close resemblance is shown by 6-*hydroxy*-2 : 2 : 4-*trimethylchroman*, synthesised from 6-hydroxy-4-methylcoumarin. Structural formulæ for the tocopherols are proposed and discussed. The degradative and synthetic evidence available does not distinguish definitely between chroman and coumaran formulæ for the tocopherols.

In Part III of this series (this vol., p. 253) it was suggested that α- and β-tocopherol were probably coumaran or chroman derivatives bearing a long side chain in the heterocyclic nucleus. With a view to test this hypothesis we undertook the synthesis of compounds of this type. Some of our results have already been reported (*Nature*, 1938, **141**, 646) and are now given in more detail.

Smith and MacMullen (*J. Amer. Chem. Soc.*, 1936, **58**, 630) showed that condensation of ψ-cumoquinone with ethyl sodioacetoacetate, followed by acidification of the initially formed addition product, yielded a mixture of 5-hydroxy-4 : 6 : 7-trimethyl*iso*coumaranone

Work, *loc. cit.*) and of duroquinol from the latter (Fernholz, *J. Amer. Chem. Soc.*, 1937, **59**, 1154) indicates that α-tocopherol differs from β-tocopherol in having an additional methyl group on the aromatic nucleus. Since 5-hydroxy-4 : 6 : 7-trimethyl-2-*n*-heptadecylcoumaran gives duroquinol on pyrolysis, it may be concluded that α-tocopherol has three such methyl groups on the aromatic nucleus. As to the nature of the side chain, it has been established that the tocopherols contain several side methyl groups in addition to those on the aromatic ring. Since the tocopherols contain 20 carbon atoms in addition to those accounted for by the aromatic nucleus, it is attractive on biogenetic grounds to regard them as built up by condensation of quinols with phytol (VII). This substance occurs in all green plants as a constituent of chlorophyll, and it is noteworthy that it also accompanies the tocopherols in wheat-germ oil (Todd, Bergel, and Work, *Biochem. J.*, 1937, **31**, 2259). On this basis, α-tocopherol would have structure (VIII) or (IX) according to whether it is a chroman or coumaran derivative.

For β-tocopherol, if it differs from the α-compound only by having one less methyl group on the aromatic ring, three isomers (derived from *o*-, *m*-, and *p*-xyloquinol) are possible, both with coumaran and chroman structures, and it is likely that synthesis will prove the best means of distinguishing between them. In Part III (*loc. cit.*) we reported the presence of traces of an unidentified higher-melting quinol in addition to ψ-cumoquinol in the pyrolytic products of β-tocopherol. There is a possibility that this may have been *p*-xyloquinol (m. p. 215°) or *o*-xyloquinol (m. p. 221°) produced by fission of the heterocyclic ring at the point of attachment to the aromatic ring. It should, of course, be possible to obtain dimethylmaleic acid by oxidation of β-tocopherol if it is derived from *o*-xyloquinol but this would require considerably larger amounts of material than are at our disposal.

The conclusion that α-tocopherol might possess structure (VIII) was independently reached by Fernholz (*J. Amer. Chem. Soc.*, 1938, **60**, 700), and somewhat later the view that the tocopherols were coumarans or chromans with long side chains was also advanced by John (*Z. physiol. Chem.*, 1938, **252**, 222) and by Karrer, Salomon, and Fritzsche (*Helv. Chim. Acta*, 1938, **21**, 309). Fernholz favoured structure (VIII) rather than (IX) for α-tocopherol because he obtained on chromic acid oxidation a γ-lactone, $C_{21}H_{40}O_2$. He maintained that formation of a γ-lactone indicated a chroman structure for the vitamin, but, as Karrer, Fritzsche, Ringier, and Salomon (*ibid.*, p. 520) have pointed out, it is possible to derive the lactone $C_{21}H_{40}O_2$ from a coumaran structure also, so the evidence is not conclusive. Recently, John, Dietzel, Günther, and Emte (*Naturwiss.*, 1938, **26**, 366) have also brought forward evidence in support of a chroman structure for the vitamin, but it seems as though a final decision will be most readily attained by complete and unambiguous synthesis.

Before the appearance of Fernholz's publication (*loc. cit.*) we had carried out some further oxidations on β-tocopherol. Our quantities of material were too small to permit of the isolation of any identifiable products other than a fatty acid, apparently $C_{12}H_{24}O_2$; in view of Fernholz's results we have not continued the investigation. The results of catalytic hydrogenation of α-tocopherol are also recorded in the experimental section; it absorbs 4 mols. of hydrogen in the same way as β-tocopherol (Part III; *loc. cit.*).

Experimental.

Ethyl Stearoylacetoacetate.—To sodium dust (2·5 g.) covered with dry ether (400 c.c.), ethyl acetoacetate (28·2 g.; 2 mols.) diluted with dry ether (100 c.c.) was gradually added, and the mixture set aside overnight. Stearoyl chloride (32·7 g.) (Izar, *Biochem. Z.*, 1912, **40**, 403), dissolved in ether (100 c.c.), was added fairly rapidly at room temperature, and the whole subsequently refluxed for 1 hour in a nitrogen atmosphere, sodium chloride separating. After cooling, the mixture was diluted with water, and the ethereal layer tapped off and dried. After removal of ether and excess ethyl acetoacetate by distillation, the residue crystallised from alcohol. After two recrystallisations, the *ester* had m. p. 42° (Found: C, 72·7; H, 11·4. $C_{24}H_{44}O_4$ requires C, 72·7; H, 11·1%). Yield 29 g.

Ethyl Stearoylacetate.—The above ester (23 g.), suspended in water (300 c.c.) containing sodium hydroxide (2·3 g.), was heated on the water-bath during 45 minutes, and the milky liquid quickly cooled and extracted with ether. After being washed successively with sodium

carbonate solution and water, the ethereal solution was dried over sodium sulphate and evaporated. The oily residue set to a mass of colourless crystals. After six recrystallisations from alcohol, these had m. p. 46·5° (Found : C, 74·7; H, 11·8. $C_{22}H_{42}O_3$ requires C, 74·5; H, 11·8%). Yield 15 g. On addition of ammoniacal copper acetate to an alcoholic solution the copper salt separated as pale green crystals, m. p. 111—112°.

Methyl n-*Heptadecyl Ketone.*—To ethyl stearoylacetate (0·5 g.) dissolved in alcohol (10 c.c.), sodium hydroxide solution (20 c.c. of 2N) was added, and the mixture boiled under reflux for 12—14 hours. On diluting with water, extracting with ether and evaporating the ethereal solution, an oil was obtained which solidified on standing. Recrystallised from alcohol, the *ketone* formed colourless platelets, m. p. 57°. Despite repeated trials satisfactory analytical values could not be obtained (Found : C, 80·1; H, 12·9. $C_{19}H_{38}O$ requires C, 80·8; H, 13·5%). The *semicarbazone* crystallised from methyl alcohol as needles, m. p. 124—125° (Found : N, 12·2. $C_{20}H_{41}ON_3$ requires N, 12·4%), and from it the ketone, m. p. 57°, could be readily regenerated.

Condensation of ψ-Cumoquinone with Ethyl Sodiostearoylacetate.—To the ester (3·5 g.) in absolute alcohol (150 c.c.), a solution of sodium (0·23 g.) in absolute alcohol (25 c.c.) was added at room temperature. A solution of ψ-cumoquinone (1·5 g.) in absolute alcohol (25 c.c.) was dropped slowly into the mixture, which was frequently shaken during the addition. The ethyl sodiostearoylacetate slowly dissolved, and after standing overnight the purple-red solution was poured on a mixture of crushed ice (300 g.) and concentrated hydrochloric acid (9·4 c.c.). The brown precipitate, initially resinous, soon solidified, and was collected and dissolved in hot alcohol to which were added a few drops of alcoholic hydrogen chloride. On cooling, 5-*hydroxy*-3-*stearoyl*-4 : 6 : 7-*trimethyl*isocoumaranone (I; R = $CO{\cdot}C_{17}H_{35}$) separated in waxy crystals. Recrystallised from benzene–light petroleum, it had m. p. 104° (1·2 g.) (Found : C, 76·1; H, 9·9. $C_{29}H_{46}O_4$ requires C, 76·0; H, 10·0%). The substance reduced neutral silver nitrate fairly readily on heating, was soluble in aqueous sodium hydroxide, and gave a blue colour with ferric chloride in alcoholic solution.

The alcoholic mother-liquors from the first crystallisation of the above substance yielded on concentration small amounts of 5-hydroxy-4 : 6 : 7-trimethyl-2-*n*-heptadecylcoumarone (II; R = $C_{17}H_{35}$), m. p. 101—102° (see below), and 5-*hydroxy*-4 : 6 : 7-*trimethyl*-2-n-*heptadecylcoumarone*-3-*carboxylic acid* (III). The latter, recrystallised from benzene–light petroleum, formed a colourless crystalline powder, m. p. 158—159° (Found : C, 76·1; H, 9·8. $C_{29}H_{46}O_4$ requires C, 76·0; H, 10·0%). It was soluble in dilute sodium carbonate solution, and on heating to 230—240°/14 mm. during 2 hours it lost carbon dioxide, yielding (II; R = $C_{17}H_{35}$), identified by m. p. and mixed m. p.

5-*Hydroxy*-4 : 6 : 7-*trimethyl*-2-n-*heptadecylcoumarone* (II; R = $C_{17}H_{35}$).—(A) The *iso*coumaranone (I; R = $CO{\cdot}C_{17}H_{35}$) (0·5 g.) was dissolved in a mixture of absolute alcohol (10 c.c.) and concentrated hydrochloric acid (2·5 c.c.), and after addition of zinc dust (1·5 g.), the mixture was heated on the water-bath during 5 hours, cooled, diluted with water, and extracted with ether, the extract being washed with sodium carbonate solution and dried over sodium sulphate. The oily residue left on evaporation of the ether quickly solidified. On dissolution in hot alcohol and cooling, the *coumarone* separated as a colourless crystalline powder. After recrystallisation from methyl or ethyl alcohol it had m. p. 101—102° (Found : C, 80·8; H, 10·9. $C_{28}H_{46}O_2$ requires C, 81·2; H, 11·1%) (yield 0·2 g.). Insoluble in sodium hydroxide solution (2N), it gave with concentrated sulphuric acid a deep yellow, and with a mixture of concentrated sulphuric and glacial acetic acids a green colour. It reduced neutral silver nitrate on warming for 5 minutes on the water-bath; under the same conditions it reduced ammoniacal silver nitrate almost instantaneously. In alcoholic solution its absorption spectrum showed maxima at 2930 A. ($\epsilon_{mol.}$ = 4554) and 2550 A. ($\epsilon_{mol.}$ = 18216).

On keeping the alcoholic mother-liquors of the above substance for a few days, *ethyl* 5-*hydroxy*-4 : 6 : 7-*trimethyl*-2-n-*heptadecylcoumarone*-3-*carboxylate* separated as colourless needles. Recrystallised from methyl alcohol, it had m. p. 68—69° (Found : C, 76·5; H, 10·3. $C_{31}H_{50}O_4$ requires C, 76·5; H, 10·3%). On hydrolysis with alcoholic potassium hydroxide (20%), the ester yielded an acid, m. p. 158—159° undepressed on admixture with (III).

(B) The above *iso*coumaranone (I; R = $CO{\cdot}C_{17}H_{35}$) (1 g.) was refluxed for 5 hours with a mixture of glacial acetic acid (40 c.c.), concentrated hydrochloric acid (20 c.c. of 25%), and zinc dust (3 g.). After cooling, the mixture was diluted with water, washed thoroughly with sodium bicarbonate solution, dried, and evaporated. The colourless crystalline residue was recrystallised from alcohol and then had m. p. 101—102° (0·9 g.), undepressed on admixture with the coumarone (II; R = $C_{17}H_{35}$).

5-Hydroxy-4 : 6 : 7-trimethyl-2-n-heptadecylcoumaran (IV).—The above coumarone (0·6 g.), dissolved in glacial acetic acid (*ca.* 75 c.c.), was shaken at 46—47° with hydrogen in presence of palladised charcoal (*ca.* 0·4 g.) during 3 hours. Hydrogen absorption was then complete, the total amount absorbed corresponding approximately to 1 mol. After being filtered, the solution was diluted with water, extracted with ether, and the extracts, after being washed thoroughly with water and sodium bicarbonate, were dried and evaporated. The residue, which solidified on standing, crystallised from methyl alcohol in colourless waxy flakes, m. p. 95—95·5° (Found : C, 81·1; H, 11·6. $C_{28}H_{48}O_2$ requires C, 80·8; H, 11·5%). The coumaran, like α- and β-tocopherol, reduced neutral silver nitrate fairly readily on warming, and gave a bright yellow colour with a mixture of concentrated sulphuric and glacial acetic acids. On treatment with cyanic acid in benzene solution at 0°, it gave a colourless *allophanate*, which, recrystallised from acetone, had m. p. 192° (Found : C, 71·9; H, 9·8; N, 5·7. $C_{30}H_{50}O_4N_2$ requires C, 71·7; H, 10·0; N, 5·6%).

Pyrolysis of (IV).—The above coumaran (80 mg.) was heated in a nitrogen atmosphere at 360—365° during 6 hours. The semi-solid distillate was washed with light petroleum (b. p. 100—120°), and the sparingly soluble crystalline residue was purified by sublimation and by recrystallisation from this solvent. The product, of which only a few mg. were available, showed the properties of duroquinol and had m. p. 220—222°; a mixture with duroquinol (m. p. 228—230°) melted at 226—228°.

Pyrolysis of (I; R = $CO{\cdot}C_{17}H_{35}$).—When the *iso*coumaranone (I; R = $CO{\cdot}C_{17}H_{35}$) was heated to 350—400° in a nitrogen atmosphere, a semi-solid distillate was obtained, and crystallisation from alcohol afforded 5-hydroxy-4 : 6 : 7-trimethyl*iso*coumaranone (I; R = H), m. p. 195—196°; mixed with an authentic specimen (m. p. 197—198°) the m. p. was 196—197°.

Condensation of ψ-Cumoquinone with Ethyl Sodiopalmitoylacetate.—To a solution of ethyl palmitoylacetate (5·8 g.) (Levene and Haller, *J. Biol. Chem.*, 1925, **63**, 669) in absolute alcohol (10 c.c.), sodium (0·41 g.) dissolved in absolute alcohol (30 c.c.) was added. To the stiff soap formed, more alcohol was added (*ca.* 100 c.c.), and to the suspension ψ-cumoquinone (2·7 g. dissolved in 30 c.c. alcohol) was added at room temperature in small quantities with frequent shaking. The suspended matter passed into solution, and after standing overnight, the red-violet liquid was poured on a mixture of crushed ice (400 g.) and concentrated hydrochloric acid (15 c.c.). The brown precipitate was collected, dissolved in hot alcohol and cooled, whereupon *5-hydroxy-3-palmitoyl-4 : 6 : 7-trimethylisocoumaranone* (I; R = $CO{\cdot}C_{15}H_{31}$) separated. Recrystallised from alcohol containing a trace of hydrogen chloride, it had m. p. 104° (Found : C, 75·4; H, 9·6. $C_{27}H_{42}O_4$ requires C, 75·6; H, 9·8%). The substance was soluble in dilute sodium hydroxide and gave a blue colour with ferric chloride in alcoholic solution. On pyrolysis at 350—400° it gave, together with unidentified products, 5-hydroxy-4 : 6 : 7-trimethyl*iso*coumaranone (I; R = H), identified by m. p. and mixed m. p.

The alcoholic mother-liquors of the above *iso*coumaranone were subjected to steam-distillation, which removed some unchanged quinone as well as alcohol. The residue was taken up in ether, and the ethereal solution dried and evaporated. The oil obtained was dissolved as far as possible in light petroleum (b. p. 40—60°) and subjected to chromatographic analysis on activated aluminium oxide (Merck), the column being developed with a mixture of light petroleum (b. p. 40—60°) and benzene (10 : 1). The chromatogram showed several indistinct bands in the upper part of the column, from which only resinous material could be obtained, but the lowest portion, which had a distinct blue fluorescence when viewed in ultra-violet light, yielded on elution with ether–methyl alcohol (1 : 4) a small amount of *5-hydroxy-4 : 6 : 7-trimethyl-2-n-pentadecylcoumarone* (II; R = $C_{15}H_{31}$). Recrystallised from methyl alcohol, it had m. p. 100—101° (Found : C, 80·6; H, 10·7. $C_{26}H_{42}O_2$ requires C, 80·8; H, 10·9%). The substance reduced neutral silver nitrate slowly on warming, and resembled the corresponding *n*-heptadecyl compound (II; R = $C_{17}H_{35}$) closely in its other properties. In alcoholic solution its absorption spectrum showed maxima at 2950 A. ($\epsilon_{mol.}$ = 3474) and 2550 A. ($\epsilon_{mol.}$ = 18914).

5-Hydroxy-4 : 6 : 7-trimethyl-2-n-pentadecylcoumarone (II; R = $C_{15}H_{31}$).—Treatment of the *iso*coumaranone (I; R = $CO{\cdot}C_{15}H_{31}$) (0·3 g.) with zinc and alcoholic hydrochloric acid in the manner already described for the corresponding heptadecyl compound yielded the coumarone (0·1 g.), m. p. 103—104° (Found : C, 80·5; H, 10·7%). Mixed with the above-described product, it had m. p. 102—104°. From the alcoholic mother-liquors of this substance, a second crystalline product (0·1 g.) was obtained, m. p. 63° to an opaque liquid which cleared at 71°. From analysis and mode of preparation it was considered to be *ethyl 5-hydroxy-4 : 6 : 7-trimethyl-2-n-pentadecylcoumarone-3-carboxylate* (Found : C, 75·9; H, 10·2. $C_{29}H_{46}O_2$ requires C, 76·0; H, 10·0%).

ψ-Cumoquinol Monoallyl Ether.—ψ-Cumoquinol (0·7 g.) was added to powdered potassium (0·2 g.) in toluene (20 c.c.), and the mixture refluxed in a hydrogen atmosphere for 30 mins. Ethyl alcohol (0·5 g.) was added, and the mixture refluxed until no more potassium remained. Allyl bromide (1·5 mol.) was added to the black gel, and the mixture refluxed for 2½ hours. Unchanged quinol was removed by pouring the product into aqueous sodium hyposulphite, extracting with ether, and separating the extract into fractions soluble and insoluble in light petroleum. The petroleum-soluble oil was distilled under 0·5 mm., the fraction of b. p. 110—120° being O-*monoallyl ψ-cumoquinol* (yield 40%) (Found : C, 75·1; H, 8·3. $C_{12}H_{16}O_2$ requires C, 75·0; H, 8·3%).

5-*Hydroxy*-2 : 4 : 6 : 7-*tetramethylcoumaran.*—(A) The above allyl ether (0·4 g.) was heated at 230° for 1½ hours. The trimethylallylquinol so formed was not purified but mixed with pyridine hydrochloride (1 g.) and heated at 210° for a further hour. The product was extracted with ether, washed with sulphuric acid, and the extracted oil steam-distilled. The colourless crystalline *coumaran* distilling over was recrystallised from light petroleum (b. p. 60—80°); m. p. 128—129° (Found : C, 74·6; H, 8·1. $C_{12}H_{16}O_2$ requires C, 75·0; H, 8·3%) (yield 0·1 g.).

(B) ψ-Cumoquinol (0·25 g.) was refluxed for 3 days with allyl bromide (1 mol.) and zinc chloride (0·25 g.) in a mixture of equal parts of carbon disulphide and carbon tetrachloride (25 c.c.). Unchanged quinol was removed by pouring into petroleum (60—80°) and keeping the mixture overnight at 0°. The petroleum solution was filtered, evaporated, and the residual oil steam-distilled. The coumaran distilling over recrystallised from light petroleum (b. p. 60—80°), m. p. 128—129° (yield 30 mg.).

p-*Xyloquinol Monoallyl Ether.*—*p*-Xyloquinol (1·38 g.) was dissolved in ethyl alcohol (25 c.c.), and potassium (0·39 g.) added. The mixture was refluxed for ½ hour in hydrogen, and allyl bromide (1·21 g.) added. After refluxing for a further 2½ hours, the mixture was poured into water, extracted with ether, and the extracted oil distilled under 0·5 mm. The fraction, b. p. 115—120°, was p-*xyloquinol monoallyl ether* (Found : C, 73·8; H, 7·9. $C_{11}H_{14}O_2$ requires C, 74·2; H, 7·8%) (yield 0·73 g.).

6-*Hydroxy*-2 : 2 : 4-*trimethyl*-Δ^3-*chromen.*—6-Acetoxy-4-methylcoumarin (5·9 g.) (Borsche, *Ber.*, 1907, **40**, 2732), dissolved in dry benzene (250 c.c.), was gradually added to a solution of methylmagnesium iodide [prepared from magnesium (11·4 g.) and methyl iodide (67 g.) in ether–benzene], the whole being cooled in ice. After removal of ether by distillation, the mixture was refluxed for 2 hours, cooled, and treated with crushed ice and hydrochloric acid. The benzene layer was tapped off and evaporated, leaving a colourless oil which crystallised rapidly. Recrystallised from light petroleum (b. p. 60—80°), 6-*hydroxy*-2 : 2 : 4-*trimethyl*-Δ^3-*chromen* was obtained as faintly yellowish prisms, m. p. 104—105° (Found : C, 75·8; H, 7·3. $C_{12}H_{14}O_2$ requires C, 75·8; H, 7·4%). The acetyl group present in the initial material seems to be completely removed in working up, since the crude product, m. p. 102—104°, was unaffected by treatment with alkali. The substance gave a deep cherry-red colour with concentrated sulphuric acid alone or mixed with glacial acetic acid, and reduced neutral silver nitrate readily on warming. 6-Hydroxy-4-methylcoumarin gives practically no colour with sulphuric acid and does not reduce silver nitrate save in presence of ammonia. In alcoholic solution the absorption spectrum of the chromen had a maximum at 3340 A. ($\epsilon_{mol.} = 3724$).

6-*Hydroxy*-2 : 2 : 4-*trimethylchroman* (VI).—The above chromen (0·5 g.), dissolved in methyl alcohol (50 c.c.), was shaken with hydrogen at room temperature in presence of palladised charcoal (1 g.). After 30 minutes, hydrogen absorption had ceased, the volume absorbed being 65 c.c. (Calc. for 1 mol. : 66 c.c.). The mixture was filtered, the methyl-alcoholic solution evaporated, and the residue recrystallised from light petroleum (b. p. 60—80°), affording colourless needles, m. p. 107—108° (Found : C, 74·8; H, 8·2. $C_{12}H_{16}O_2$ requires C, 75·0; H, 8·3%). The *compound* gave a bright yellow colour with a mixture of concentrated sulphuric and glacial acetic acids, and reduced neutral silver nitrate readily on warming. Treatment with cyanic acid in benzene solution yielded an allophanate, m. p. 182°.

Oxidation of β-*Tocopherol.*—β-Tocopheryl allophanate (350 mg.) was hydrolysed by boiling for 1 hour with 5% methyl-alcoholic potassium hydroxide; the product was dissolved in methanol (25 c.c.), silver nitrate (1 g.) added, and the solution heated on the water-bath for 1 hour, diluted with water, and extracted with ether. The deep red ethereal solution of the quinone so obtained was completely decolorised with sodium hyposulphite solution. The colourless oil was allowed to stand with excess diazomethane in ether for 12 hours, and the methylated product treated with a mixture of potassium hydrogen sulphate (100 mg.) and chromic acid (225 mg.; 5 mols.) in glacial acetic acid (25 c.c.) and warmed on the water-bath until oxidation was complete. The crude mixture was steam-distilled, the distillate extracted with ether,

the ether freed from acetic acid by washing with water, and the extract separated into a neutral and an acid fraction. The oily acid fraction possessed a strong smell of a lower fatty acid, but an attempt to prepare a crystalline anilide was unsuccessful. The neutral volatile fraction gave, on crystallisation from acetone, a trace of a compound, m. p. 50—52°, and a micro-Zeisel determination indicated 10·5% OCH_3. There was insufficient material for complete purification and identification.

The non-steam-volatile oxidation products were also separated into neutral and acid fractions, and the former, which corresponded in weight to about 50% of the starting material, was further oxidised with chromic acid (225 mg.) and worked up as before. The combined non-volatile acid (90 mg.) was distilled in a high vacuum. There was considerable decomposition, but 31 mg. of a colourless acid were collected (Found, by titration with NaOH: *M*, 192. Calc. for $C_{12}H_{24}O_2$: *M*, 200). The acid gave a crystalline *p*-phenylphenacyl ester, m. p. 98—100° (Found: C, 79·2; H, 8·2. Calc. for $C_{26}H_{34}O_3$: C, 79·2; H, 8·6%).

Micro-hydrogenations.—These determinations were carried out by Mr. F. Boston, of Manchester University, using platinum oxide as catalyst. (I) α-Tocopherol (2·675 mg.) absorbed 0·5453 c.c. (3·9 mols.) of hydrogen at *N.T.P.*, the solvent being glacial acetic acid. No absorption occurred in the cold, but slow and regular absorption took place at 90°. (II) α-Tocopheryl allophanate (2·94 mg.) absorbed 0·5220 c.c. (4·09 mols.) of hydrogen at *N.T.P.*, the solvent being decalin–acetic acid; 1 mol. was absorbed in the cold, and the other 3 mols. on heating to 90° for 8 hours.

The biological tests were carried out by Miss A. M. Copping under a grant from the Medical Research Council, to whom we are indebted. We also thank Messrs. Glaxo Laboratories Ltd., and Messrs. Hoffmann-La Roche and Co., for their generous assistance.

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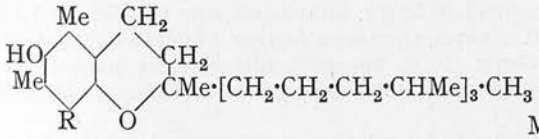
258. *Studies on Vitamin E. Part V. Synthesis of Racemic α-Tocopherol and of a Lower Homologue.*

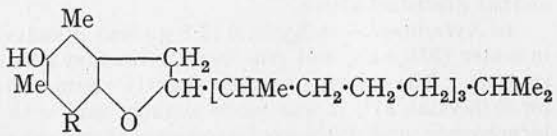
By F. Bergel, (Miss) A. M. Copping, (Miss) A. Jacob, A. R. Todd, and T. S. Work.

Racemic α-tocopherol may be synthesised by condensation of phytol with ψ-cumoquinol. When *m*-xyloquinol is used, a product is obtained which also has vitamin E activity and may be isomeric or identical with racemic β-tocopherol. The synthetic method does not, however, distinguish between a chroman or a coumaran type of structure for the tocopherols.

In the preceding paper we proposed for α-tocopherol the structure (I; R = Me) or (II; R = Me) (cf. also Fernholz, *J. Amer. Chem. Soc.*, 1938, **60**, 700; Karrer, Salomon, and Fritzsche, *Helv. Chim. Acta*, 1938, **21**, 309), β-tocopherol being regarded as differing from the α-compound by having only two methyl groups on the aromatic nucleus. Recently, Karrer, Fritzsche, Ringier, and Salomon (*ibid.*, p. 520) have reported the first synthesis of a substance having structure (I; R = Me) or (II; R = Me) by heating together phytyl bromide and ψ-cumoquinol in presence of zinc chloride, and they have now shown (*Nature*, 1938, **141**, 1057) that this material, giving an allophanate m. p. 172°, is indeed racemic α-tocopherol, since they have been able to resolve it by means of *d*-bromocamphorsulphonic acid and have obtained a product which they state is identical with the natural vitamin.



(I.)



(II.)

As announced in a preliminary note (Bergel, Jacob, Todd, and Work, *Nature*, 1938, **142**, 36), we have synthesised racemic α-tocopherol by a rather simpler method than that used above. Phytol can be directly condensed with ψ-cumoquinol by heating a mixture of the two substances, preferably in decalin solution, in presence of zinc chloride. The product has high vitamin E activity, but we have not yet resolved it into optical isomers. This synthesis, although fairly satisfactory as regards yield of product, is, like that of the Swiss workers, unsatisfactory in that it fails to distinguish between formulæ (I; R = Me) and (II; R = Me) for α-tocopherol. Karrer and his colleagues believe that their synthetic product has structure (II; R = Me) on the grounds that allyl bromide yields coumarans when condensed with phenols in presence of zinc chloride. Since phytyl bromide, however, is a γγ-dialkylallyl bromide, it seems to us that the argument is not necessarily valid, and it may well be that the synthetic product has a chroman structure. We are now engaged on the synthesis of the tocopherols by unequivocal methods in order to solve the problem finally. According to the view expressed in Part IV (preceding paper), β-tocopherol might have one of three isomeric structures derived severally from *o*-, *m*-, and *p*-xyloquinol and phytol. When phytol is condensed with *m*-xyloquinol, the product has properties similar to those of β-tocopherol. The condensation seems more difficult than in the case of ψ-cumoquinol. This product, which should have structure (I; R = H) or (II; R = H), has high vitamin E activity, and yields a crystalline *allophanate*, m. p. 148—149°, but whether or not it represents a racemic form of β-tocopherol can only be decided by resolution and by synthesis of the corresponding substances from *o*- and *p*-xyloquinol.

In preliminary experiments both of these synthetic " tocopherols " have shown a high degree of vitamin E activity, but it is not yet possible to state the minimum active dose. A series of biological experiments to this end is being made by one of us (A. M. C.), and the results will be reported elsewhere.

Experimental.

Condensation of Phytol with ψ-Cumoquinol.—(A). A mixture of phytol (1·0 g.), ψ-cumoquinol (0·7 g.), and anhydrous zinc chloride (0·3 g.) was heated rapidly to 180—190° and kept at this temperature during 15 minutes. The melt became brown, and a certain amount of ψ-cumoquinol sublimed out of the mixture. After cooling, the mixture was shaken with light petroleum (50 c.c.; b. p. 60—80°), decanted from zinc chloride, left overnight, filtered, and submitted to chromatographic analysis on activated aluminium oxide (Merck), being developed with light petroleum (b. p. 60—80°). The narrow brownish layer at the top of the column was discarded, as was the purplish lowest layer, and the broad middle portion, which was nearly colourless, was eluted with benzene–acetone–methyl alcohol (8 : 1 : 1). The yellowish oil obtained (200 mg.) reduced neutral silver nitrate on warming, and gave a bright yellow colour with a mixture of concentrated sulphuric and glacial acetic acids. On pyrolysis at about 360°, a crystalline product, m. p. *ca.* 215°, was obtained having the properties of duroquinol; oxidation with ferric chloride gave a quinone, m. p. 101—103°, a mixture of which with duroquinone (m. p. 106—108°) melted at 104—106°.

Treatment with cyanic acid in benzene solution gave an allophanate, m. p. 169—170°, identified by mixed m. p. (169—171°) with the allophanate (m. p. 170—171°) obtained by following the method of Karrer, Fritzsche, Ringier, and Salomon (*loc. cit.*) (Found: C, 71·9; H, 10·1; N, 5·3. Calc. for $C_{31}H_{52}O_4N_2$: C, 72·0; H, 10·2; N, 5·4%). The absorption spectrum of the allophanate (max. 2860 A.; min. 2520 A.) was almost identical with that of α-tocopheryl allophanate, and that of the oily alcohol obtained on hydrolysis (max. 2980 A.; min. 2600 A.) was almost identical with that of α-tocopherol itself.

(B). A mixture of phytol (1 g.), ψ-cumoquinol (0·52 g.), anhydrous zinc chloride (0·5 g.), and decalin (10 c.c.) was heated to 200° for 30 minutes, then for a further 4 hours at 160—180°; it was then cooled, diluted with light petroleum (b. p. 60—80°), filtered, and subjected to chromatographic analysis as above. The oil obtained (350 mg.) gave the same allophanate as that described above.

m-*Xyloquinol.*—*m*-Xylenol (7·8 g.) was dissolved in a solution of sodium hydroxide (12·5 g.) in water (375 c.c.), and powdered potassium persulphate (15 g.) added in small portions with shaking. The solution became slightly warm and developed a brown colour. After standing for 2 days at 37°, it was made slightly acid with hydrochloric acid, neutralised with sodium bicarbonate, and unchanged *m*-xylenol blown off with steam. The residue was made strongly acid, boiled for a few minutes, cooled, extracted thoroughly with ether, and the extracts dried

and evaporated. The solid residue, recrystallised from xylene, gave *m*-xyloquinol, m. p. 150—151° (lit., m. p. 149°).

Condensation of Phytol with m-*Xyloquinol.*—Phytol (1 g.), *m*-xyloquinol (0·46 g.), decalin (7·0 c.c.), and anhydrous zinc chloride (0·5 g.) were refluxed gently during 6 hours, a further amount (0·5 g.) of zinc chloride being added after 3 hours' heating, and worked up in the manner employed in the ψ-cumoquinol condensation. The product was a yellowish oil (450 mg.), which reduced neutral silver nitrate on warming and gave a bright yellow colour with a mixture of concentrated sulphuric and glacial acetic acids. On pyrolysis at about 360°, it gave a crystalline distillate, m. p. 168—170°, identified as ψ-cumoquinol by m. p. and mixed m. p. Treatment with cyanic acid in benzene solution gave an allophanate, m. p. 148—149°, having an absorption spectrum (max. 2860 A.; min. 2520 A.) closely similar to that of the tocopheryl allophanates and giving on hydrolysis an oily alcohol spectroscopically (max. 2980 A.; min. 2620 A.) very similar to the tocopherols. The same *allophanate*, m. p. and mixed m. p. 148—149°, was obtained by substituting *m*-xyloquinol for ψ-cumoquinol in the process used by the Swiss workers (*loc. cit.*) (Found : C, 71·8; H, 10·1; N, 5·4. $C_{30}H_{50}O_4N_2$ requires C, 71·7; H, 10·0; N, 5·6%).

We have to thank Messrs. Hoffmann-La Roche & Co. and Messrs. Glaxo Laboratories Ltd. for their assistance, and the Medical Research Council for a grant to one of us (A. M. C.).

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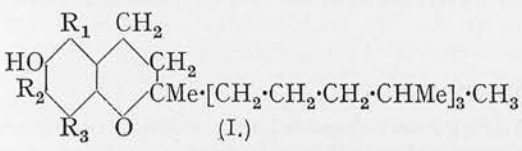
123. *Studies on Vitamin E. Part VI. Synthesis of Lower Homologues of α-Tocopherol.*

By Anni Jacob, Marguerite Steiger, A. R. Todd, and T. S. Work.

In continuation of the work described in Part V (J., 1938, 1382) two racemic tocopherols (I; $R_1 = R_3 = Me$; $R_2 = H$) and (II; $R_1 = H$; $R_2 = R_3 = Me$) have been synthesised. Satisfactory yields of these are obtained by condensing p- and o-*xyloquinol monobenzoates* with phytol or phytyl bromide in presence of zinc chloride. The products are characterised as crystalline p-*nitrophenylurethanes* and show biological activity comparable with that of β- and γ-tocopherol.

It is now generally accepted that α-tocopherol, the most active anti-sterility factor (vitamin E) known to occur in nature, is to be regarded as 6-hydroxy-2 : 5 : 7 : 8-tetramethyl-2-(4′ : 8′ : 12′-trimethyltridecyl)chroman (I; $R_1 = R_2 = R_3 = Me$). Its formulation as a chroman rather than a coumaran derivative is justified on the basis of certain degradative evidence (Fernholz, *J. Amer. Chem. Soc.*, 1938, **60**, 700; John, Dietzel, Günther, and Emte, *Naturwiss.*, 1938, **26**, 366) and by the methods used for its synthesis (Karrer, Salomon, and Fritzsche, *Helv. Chim. Acta*, 1938, **21**, 520; Bergel, Copping, Jacob, Todd, and Work, J., 1938, 1382; Smith, Ungnade, and Pritchard, *Science*, 1938, **88**, 37).



(I.)

The naturally occurring anti-sterility factors β- and γ-tocopherol are to be regarded as isomeric lower homologues of α-tocopherol bearing only two methyl groups on the aromatic nucleus (cf. Part V, *loc. cit.*), the side chain in both being the same as in α-tocopherol (Emerson, *J. Amer. Chem. Soc.*, 1938, **60**, 1741). In Part V it was pointed out that three isomers fulfilling these conditions were possible, derived from *o*-, *m*-, and *p*-xyloquinol, and one of these was synthesised by condensation of phytol with *m*-xyloquinol. The tocopherol had a vitamin E activity roughly comparable with those of β- and γ-tocopherol. Consequent on this observation we undertook the synthesis of the remaining isomers. Direct condensation of *o*- or *p*-xyloquinol with phytol or phytyl bromide was not a practicable method; the products were complex mixtures and separation of the pure tocopherols was extremely difficult. Karrer and Fritzsche (*Helv. Chim. Acta*, 1938, **21**, 1234) recorded rather similar results; by direct condensation of the xyloquinols with phytol or phytyl bromide, followed by chromatographic analysis, they obtained vitamin E-active oils, which, although they gave analytical figures approximating to the calculated values for $C_{28}H_{48}O_2$, were apparently impure, since the Swiss workers failed to obtain pure derivatives from them.

Our initial approach to the synthesis of the desired substances was by condensation of the *quinol monobenzyl ethers* with phytyl bromide, since the benzyl group, which would prevent condensation with two phytyl residues, could be removed subsequently by catalytic hydrogenation. Using this method, we obtained oils showing vitamin E activity, but the products were difficult to purify and the yields rather low. A further inconvenience lay in the fact that the benzyl ethers were difficult to prepare in good yield. A variety of methods were tried for their preparation, but none was really satisfactory. An interesting feature of the direct benzylation of *p*-xyloquinol was the formation of a violet-red *substance*, m. p. 111—112°, as a by-product. This substance was not further investigated, but analysis and absorption spectrum suggest that it may be a derivative of a diquinone analogous to the diduroquinone of v. Pechmann (*Ber.*, 1889, **22**, 2115).

Condensation of the *monobenzoates* with phytol or phytyl bromide, followed by the removal of benzoyl groups by hydrolysis, proved entirely satisfactory, chromatographic analysis of the products yielding the desired tocopherols as yellowish oils showing full vitamin E activity in rats at a dose of 10 mg. From both of them, crystalline p-*nitrophenylurethanes* were readily prepared. All these synthetic tocopherols are, of course, racemic about carbon atom 2 in the heterocyclic ring and have not yet been resolved. The product from *p*-xyloquinol (I; $R_1 = R_3 = Me$; $R_2 = H$) shows vitamin E activity

in a dose of 5 mg., which corresponds to the minimum active dose of natural β-tocopherol (cf. Part II, *Biochem. J.*, 1937, **31**, 2257); the minimum active dose of the others is at present being determined.

John, Günther, and Schmeil (*Ber.*, 1938, **71**, 2637) have described experiments carried out in the course of their efforts to synthesise α-tocopherol and simpler 6-hydroxychroman derivatives. We record here briefly some results of experiments, carried out early in 1938, which more or less confirm the observations of the German workers. In an endeavour to synthesise 6-hydroxy-2 : 2 : 5 : 7 : 8-pentamethylchroman we condensed ψ-cumoquinol with ββ-dimethylacrylyl chloride in the presence of aluminium chloride; the only crystalline product was a small amount of a substance, m. p. 232°, spectroscopically unlike a chromanone. Similarly we failed to isolate any chromanone in a similar condensation of phytenyl chloride with ψ-cumoquinol. Among other unsuccessful attempts to synthesise α-tocopherol was an endeavour to condense 3-bromophytan 1-acetate with ψ-cumoquinol in the presence of sodium ethoxide, followed by removal of the acetyl residue and ring closure; no pure products could be isolated.

Experimental.

p-*Xyloquinol Benzyl Ethers.*—*p*-Xyloquinol (30 g.) was dissolved in a solution of sodium (4·9 g.; 1 atom) in absolute alcohol (150 c.c.), benzyl chloride (27·4 g.) added, and the mixture refluxed for 4 hours in a nitrogen atmosphere. After dilution with ether and filtration from sodium chloride the solution was evaporated, and the residue dissolved in the minimum quantity of hot methyl alcohol. On cooling, p-*xyloquinol dibenzyl ether* separated in colourless needles (12 g.); recrystallised from methyl alcohol, it had m. p. 130° (Found: C, 82·9; H, 6·6. $C_{22}H_{22}O_2$ requires C, 83·0; H, 6·9%).

The combined methyl-alcoholic mother-liquors were evaporated, and the residue extracted with light petroleum (b. p. 40—60°); the extract was allowed to flow through a column of activated aluminium oxide (Merck), the column being then washed with more light petroleum. From the filtrate and washings a further amount of dibenzyl ether (2 g.) was obtained. The residue of the light petroleum extraction was now dissolved in benzene, filtered from *p*-xyloquinol (5 g.), and poured through the same column as was used above. The chromatogram formed was washed with benzene until nothing more came through. Unchanged *p*-xyloquinol remained adsorbed and could be eluted with ether. The benzene filtrate and washings, which were yellow, were concentrated to small bulk and diluted with light petroleum. On standing, two compounds separated simultaneously, one forming warty aggregates of colourless prisms (A) and the other rosettes of violet-red needles (B). These were separated mechanically. (A) on recrystallisation from ligroin gave p-*xyloquinol monobenzyl ether* (3·5 g.), colourless prisms, m. p. 92—93° (Found: C, 78·6; H, 6·9. $C_{15}H_{16}O_2$ requires C, 78·9; H, 7·0%). (B) crystallised from nearly colourless solutions in ligroin in violet-red needles (2 g.), m. p. 111—112° (Found: C, 76·0; H, 6·6. $C_{23}H_{24}O_4$ requires C, 75·8; H, 6·6%). This *substance*, which was quite stable, dissolved in aqueous alkali to give solutions from which it was reprecipitated with acids, and on oxidation with chromic acid gave *p*-xyloquinone together with other products. Alcoholic solutions showed two well-marked absorption maxima at 2520 A.($E^{1\%}_{1\,cm.}$ = 616) and 2920 A.($E^{1\%}_{1\,cm.}$ = 113) with a band of negligible persistence at about 4300 A. Variation in the conditions of the above experiment or the use of benzyl bromide had little effect on the yield of monobenzyl ether. Other methods tried included benzylation of *p*-xyloquinol monobenzoate, use of the potassium derivative of *p*-xyloquinol, and partial debenzylation of the dibenzyl ether with aluminium chloride.

o-*Xyloquinol Benzyl Ethers.*—Benzylation of *o*-xyloquinol (10·5 g.) in the manner described above yielded in similar fashion o-*xyloquinol dibenzyl ether* (3·5 g.), crystallising from methyl alcohol in colourless needles, m. p. 109° (Found: C, 82·7; H, 6·9%), and o-*xyloquinol monobenzyl ether* (3·5 g.), crystallising from benzene–light petroleum in colourless needles, m. p. 116° (Found: C, 78·5; H, 6·9%). No red compound was formed.

Condensation of o-*Xyloquinol Monobenzyl Ether with Phytyl Bromide.*—A mixture of phytyl bromide (3 g.), *o*-xyloquinol monobenzyl ether (2 g.), anhydrous zinc chloride (2 g.), and hexane (50 c.c.) was gently refluxed for 2½ hours in a nitrogen atmosphere, by which time evolution of hydrogen bromide had ceased. The product, dissolved in light petroleum (b. p. 40—60°), was adsorbed on a column of activated aluminium oxide (Merck), the column being washed with the same solvent. From the washings a small quantity of a yellow solid was obtained as well as an

oil. The solid separated from ligroin in yellow needles, m. p. 111° (Found : C, 82·8; H. 6·1%). In alcoholic solution it showed a single absorption band at 2630 A.($E^{1\%}_{1\,cm.}$ = 532) with a second band of negligible persistence at about 4450 A.

The material adsorded on the alumina was eluted with benzene–acetone–methyl alcohol (8 : 1 : 1), hydrogenated with a palladised charcoal catalyst to remove benzyl groups, and again submitted to chromatographic adsorption. A brownish oil was finally obtained showing vitamin E activity in rats at a dose of 30 mg. On pyrolysis it gave a poor yield of ψ-cumoquinol, m. p. 165—167°, and allophanation did not give a homogeneous product.

Condensation of p-*Xyloquinol Monobenzyl Ether with Phytyl Bromide.*—Condensation as described above gave oils showing some vitamin E activity in doses of 10 mg., but allophanation did not give a homogeneous product.

p-*Xyloquinol Benzoates.*—To a solution of *p*-xyloquinol (2 g.) in dry pyridine (10 c.c.), benzoyl chloride (3 g.; 1·5 mols.) was added, and the mixture left at room temperature for 20 hours. The crude benzoate mixture obtained on working up was dissolved in hot methyl alcohol. On cooling, p-*xyloquinol dibenzoate* (1 g.) separated; it crystallised from methyl alcohol in colourless needles, m. p. 159° (Found : C, 76·5; H, 5·4. $C_{22}H_{18}O_4$ requires C, 76·3; H, 5·2%). After separation of the dibenzoate the mother-liquor was evaporated; the residue, recrystallised from light petroleum, furnished p-*xyloquinol monobenzoate* (1·9 g.) in colourless leaflets, m. p. 162—163° (Found : C, 74·7; H, 5·6. $C_{15}H_{14}O_3$ requires C, 74·4; H, 5·8%).

o-*Xyloquinol Benzoates.*—Benzoylated as above described, *o*-xyloquinol yielded a *dibenzoate*, m. p. 182° after recrystallisation from acetone–methyl alcohol (Found : C, 76·2; H, 5·3%), and a *monobenzoate*, m. p. 174—175° after recrystallisation from acetone–light petroleum (Found : C, 74·4; H, 5·9%).

p-*Xyloquinol Acetates.*—Acetylation of *p*-xyloquinol in pyridine solution with acetic anhydride (1·5 mols.) gave the diacetate, m. p. 135° (Found : C, 64·9; H, 6·4. Calc. for $C_{12}H_{14}O_4$: C, 64·9; H, 6·3%), and the *monoacetate*, m. p. 117° (Found : C, 66·4; H, 6·5. $C_{10}H_{12}O_3$ requires C, 66·7; H, 6·7%); the yield of the latter was, however, poor.

Condensation of p-*Xyloquinol Monobenzoate with Phytol.*—A mixture of the monobenzoate (2·3 g.), phytol (3 g.), anhydrous zinc chloride (1·5 g.), and decalin (25 c.c.) was heated at 170° in an oil-bath for 2½ hours, cooled, and diluted with light petroleum (b. p. 40—60°). After several hours the solution was filtered. From the filter residue, after removal of zinc chloride, unchanged monobenzoate (1·4 g.) was recovered. The filtrate was evaporated, the decalin removed in a vacuum, and the oily residue hydrolysed by refluxing for 2 hours in a hydrogen atmosphere with methyl-alcoholic potassium hydroxide (25 c.c. of 5%). The solution was cooled and extracted with ether, and the extract dried over sodium sulphate and evaporated. The residual oil was dissolved in light petroleum (b. p. 40—60°) and allowed to flow through a column of activated aluminium oxide (Merck), the chromatogram being developed with benzene until nothing further came through. Evaporation of the filtrate gave a quantity of yellowish-brown oil having no reducing properties; it probably consisted of aliphatic material. The narrow brownish layer at the top of the column was discarded and the remainder, which was approximately uniform in colour save for a yellowish ring near the base, was cut into three parts of equal length. These were separately eluted with acetone. The lowest layer yielded an oil which had no reducing properties and was therefore not further examined. The top and the middle layer yielded respectively 450 mg. and 500 mg. of crude tocopherol, which reduced neutral silver nitrate solution on warming and gave a yellow colour with concentrated sulphuric–glacial acetic acids. On pyrolysis at about 350° they yielded ψ-cumoquinol, which was identified by m. p. and mixed m. p.

When phytyl bromide was substituted for phytol in the above experiment, and the condensation carried out by refluxing a mixture of the reactants with light petroleum (b. p. 80—100°) for 5 hours, similar results were obtained. The crude tocopherol obtained in these experiments showed full vitamin E activity in rats at a dose of 10 mg.; it was not analysed, but converted directly into the p-*nitrophenylurethane*. The oil (450 mg.) was heated at 90—100° during 1 hour with *p*-nitrophenyl *iso*cyanate (0·5 g.) in a nitrogen atmosphere. The mixture was cooled, diluted with acetone containing a drop of water to destroy any *iso*cyanate, and left for an hour or two. The mixture was now evaporated, and the urethane extracted from the residue with light petroleum (b. p. 40—60°). The extract gave on evaporation a crystalline product (550 mg.) still contaminated with *p*-nitroaniline. This could be removed either by crystallisation from methyl alcohol or by absorption from light petroleum solution on a short column of aluminium oxide; when the column was washed with benzene, the urethane was eluted much more rapidly than the amine. Recrystallised from methyl alcohol, the *p*-nitrophenylurethane of tocopherol

(I; $R_1 = R_3 = Me$; $R_2 = H$) [6-hydroxy-2 : 5 : 8-trimethyl-2-(4′ : 8′ : 12′-trimethyltridecyl)-chroman] was obtained in globular aggregates of colourless crystals (450 mg.), m. p. 111—112° (Found : C, 72·5; H, 9·0; N, 4·9. $C_{35}H_{52}O_5N_2$ requires C, 72·4; H, 9·0; N, 4·8%). In alcoholic solution the absorption spectrum showed a maximum at 3160 A.(ε mol., *ca.* 16000) and a minimum at 2500 A.

Hydrolysis of the ester (100 mg.) with 5% methyl-alcoholic potassium hydroxide yielded the pure tocopherol as a slightly yellowish oil (58 mg.) corresponding closely in chemical behaviour to natural β-tocopherol. In alcoholic solution its absorption spectrum showed a maximum at 2960 A.(ε mol., *ca.* 3600) and a minimum at 2580 A. It showed full vitamin E activity when tested in rats in doses of 5 mg.

Condensation of o-*Xyloquinol Monobenzoate with Phytyl Bromide.*—The ester (3·2 g.), phytyl bromide (4·8 g.), and anhydrous zinc chloride (2 g.) were refluxed in dry benzene (30 c.c.) for 3 hours in a hydrogen atmosphere, by which time evolution of hydrogen bromide had ceased. The mixture was diluted with ether, and the solution decanted, washed, dried, and evaporated. The residue was completely soluble in light petroleum, *i.e.*, contained no unchanged *o*-xyloquinol monobenzoate. The product was subjected to the same process of hydrolysis and chromatographic analysis as in the previous experiment. The crude tocopherol (1·2 g.) was a yellowish oil showing full vitamin E activity in rats at a dose of 10 mg. It reduced neutral silver nitrate solution on warming, gave a yellow colour with concentrated sulphuric–glacial acetic acids, and on pyrolysis at about 350° yielded ψ-cumoquinol, identified by m. p. and mixed m. p. Heated with *p*-nitrophenyl *iso*cyanate either alone or in benzene solution, the product gave the p-*nitrophenylurethane* of the tocopherol (I; $R_1 = H$; $R_2 = R_3 = Me$) [6-hydroxy-2 : 7 : 8-trimethyl-2-(4′ : 8′ : 12′-trimethyltridecyl)chroman]. Recrystallised from methyl alcohol, the ester had m. p. 100° (Found : C, 72·6; H, 9·1; N, 4·8%). In alcoholic solution the absorption spectrum showed a maximum at 3130 A.(ε mol., *ca.* 13000) and a minimum at 2530 A. Hydrolysis with 5% methyl-alcoholic potassium hydroxide yielded the pure tocopherol as a slightly yellowish oil corresponding closely in chemical behaviour to natural β-tocopherol. Alcoholic solutions showed an absorption maximum at 2970 A. (ε mol., *ca.* 3400) and a minimum at 2640 A. Tests are in progress to determine the minimum active dose of this product.

p-*Nitrophenylurethane of Tocopherol from* m-*Xyloquinol and Phytol.*—The crude tocopherol (I; $R_1 = R_2 = Me$; $R_3 = H$) [6-hydroxy-2 : 5 : 7-trimethyl-2-(4′ : 8′ : 12′-trimethyltridecyl)-chroman], prepared by condensing *m*-xyloquinol with phytol (Part V, J., 1938, 1382) and biologically active in a dose of 10 mg., was heated with *p*-nitrophenyl *iso*cyanate, and the product worked up as above described. The p-*nitrophenylurethane* had m. p. 89° after recrystallisation from methyl alcohol (Found : C, 72·4; H, 8·9; N, 5·0%). In alcoholic solution the absorption spectrum showed a maximum at 3160 A. (ε mol., *ca.* 18000) and a minimum at 2520 A.

The biological tests were carried out at the Lister Institute by Miss A. M. Copping (M.R.C. grantee), to whom we are indebted. We have also to thank Messrs. Hoffmann La Roche and Company for generous gifts of material.

(*Note added, February 27th*, 1939.) Since the above was written a paper by Karrer and Fritzsche (*Helv. Chim. Acta*, 1939, **22**, 260) has appeared in which the authors describe derivatives of products obtained by direct condensation of *m*- and *p*-xyloquinol with phytol or phytyl bromide. The data given for one of these (*p*-nitrophenylurethane of I; $R_1 = R_2 = Me$, $R_3 = H$) are in good agreement with our own, but their *p*-nitrophenylurethane of (I; $R_1 = R_3 = Me$, $R_2 = H$) is apparently impure, since it melts some 20° lower than the corresponding material prepared by us *via* the benzoate. In view of this fact the argument advanced by these workers for the disposition of methyl groups in β-tocopherol is hardly conclusive.

In biological tests now in progress on our product (I; $R_1 = R_2 = Me$, $R_3 = H$) from *m*-xyloquinol, positive results have been obtained at a dosage as low as 3 mg., *i.e.*, the substance seems to have an activity approaching that of α-tocopherol. We are therefore unable to agree with the statement of Karrer and Fritzsche (*loc. cit.*, p. 261) that the synthetic isomers of β-tocopherol are three to four times less active than α-tocopherol. Their statements on constitutional specificity must be accepted with considerable reserve, since their biological tests have not been carried out with material regenerated from pure derivatives.

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XIV. THE ACTIVE PRINCIPLES OF *CANNABIS INDICA* RESIN. I

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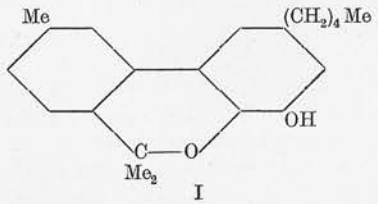
(*Received 24 November 1938*)

THE resinous exudate of the female flowers of *Cannabis indica* (*C. sativa*) forms the essential constituent of the drug variously known as hashish, bhang, charas, ganja and marihuana according to the country of origin and mode of preparation. Extracts of the flowers have been used in European medicine to some extent but were found to be so variable in activity that *C. indica* has been removed from the *British Pharmacopoeia*. The plant is, however, well known through its use as a drug in oriental countries and recently, under the name of "marihuana", its use has assumed dangerous proportions in America. *Cannabis* resin has been the subject of many investigations in the past but much of the earlier work is contradictory. The active principle is contained in a high-boiling resin and is not an alkaloid [Smith, 1857]. The active fraction isolated from the crude resin by Wood Spivey & Easterfield [1896] and given by them the name cannabinol was later found by the same workers [1899] to be a mixture, and the name cannabinol was transferred to that portion of the active resin yielding a crystalline acetate, M.P. 75°. A good deal of confusion was introduced by later workers, who, although failing to obtain a crystalline acetate, nevertheless applied the name cannabinol to their products. The situation was largely clarified by Cahn [1931], who confirmed the observations of Wood Spivey & Easterfield and established for cannabinol the formula $C_{21}H_{26}O_2$. The constitution of cannabinol has been investigated by Cahn [1930–33] and by Bergel [1932], and for it the former proposed structure (I) in which only the positions of the hydroxyl and *n*-amyl groups are to be regarded as uncertain.



I

In his last communication Cahn states that "cannabinol is not the pharmacologically active principle of *Cannabis indica*" [1933]. It seemed desirable that further investigations on the resin should be made and we have taken up the study of cannabis resin with a view to isolating the substance or substances responsible for its pharmacological action.

The starting point in these investigations was material which corresponded to the high-boiling resin (B.P. 265°/20 mm.) first described by Wood Spivey & Easterfield [1896]. It was found that cannabinol can be removed almost quantitatively as its crystalline p-*nitrobenzoate* (amounting to 25% of the whole) on

p-nitrobenzoylating the resin. On hydrolysis the crystalline ester yields cannabinol as a colourless oil, which gives the above-mentioned crystalline acetate (M.P. 75°) on acetylation. The non-crystalline portion of the *p*-nitrobenzoylated resin gave on hydrolysis a colourless oil from which no crystalline acetate could be obtained.

Active hashish preparations induce a characteristic cataleptic condition in dogs, but this effect, described first by Fraenkel [1903] is not readily made the basis of quantitative assay. Gayer [1928] showed that in various animals—e.g. cats, rabbits, mice—hashish preparations induce corneal anaesthesia and that this effect is characteristic of active fractions of the resin. This Gayer test has been developed by Marx & Eckhardt [1933] using rabbits and has been employed throughout the work described in this paper. The pure cannabinol prepared from the crystalline *p*-nitrobenzoate was found to be highly toxic when injected intravenously into rabbits but, unlike the original resin, did not produce corneal anaesthesia. The cannabinol-free resin on the other hand was much less toxic and always induced corneal anaesthesia in rabbits. This distinction is further emphasized by the fact that an acetone solution of cannabinol was found to be non-toxic after standing for 3 days in contact with air while only slight loss of activity occurred on similar treatment of the cannabinol-free resin.

Attempts to fractionate further the cannabinol-free resin by distillation or crystallization of derivatives failed completely but chromatographic analysis gave promising results. Using activated aluminium oxide as adsorbent it was possible to obtain fairly readily an oil having considerably greater activity in the Gayer test than the starting material. Even better results were obtained by submitting the oily *p*-nitrobenzoate mixture left after separating the cannabinol *p*-nitrobenzoate to chromatographic analysis on activated aluminium oxide. By this procedure remaining traces of cannabinol were removed and an oil was obtained yielding on hydrolysis a product giving a positive result in the Gayer test at a dose of 0·25 mg. per kg. body weight; this material has low toxicity as compared with cannabinol and possesses none of the convulsant action of the latter substance.

Further investigation of this highly active material is in progress and the results will be reported later. We have not as yet obtained any crystalline derivative from the most active preparation and suspect that it does not yet represent the homogeneous active principle.

Experimental

Starting material. The starting material was the resin (B.P. 185–190°/0·6 mm.) obtained on working up hashish of Indian origin in the manner described by Bergel [1930]. The resin was nearly colourless when freshly distilled. It corresponds to the "crude cannabinol" of Wood Spivey & Easterfield and possessed the characteristic pharmacological properties of the original extract.

Isolation of cannabinol. The above resin (25 g.) was dissolved in pyridine (110 ml.) and *p*-nitrobenzoyl chloride (36 g.) added. The mixture was refluxed for 4 hr., then poured on a mixture of ice and sufficient H_2SO_4 to make the resulting suspension acid to Congo red. The precipitate was collected, washed with water, dried and refluxed with light petroleum (750 ml., B.P. 80–100°) for 1 hr. and filtered hot, the filter residue being treated in the same way with a further quantity of light petroleum (250 ml.). The combined filtrates were washed with aqueous Na_2CO_3, dried and concentrated to about 150 ml. On standing *cannabinol* p-*nitrobenzoate* separated. Recrystallized first from alcohol

then from light petroleum (B.P. 80–100°) it formed pale yellow needles M.P. 160° (yield, *ca.* 6·5 g.). (Found: C, 73·0; H, 6·2; N, 3·2 %. $C_{28}H_{29}O_5N$ requires C, 73·2; H, 6·3; N, 3·2 %.) Since cannabinol *p*-nitrobenzoate is very sparingly soluble in methyl alcohol the oil left on evaporating the original light petroleum mother liquors may be largely freed of cannabinol by fractionation with this solvent.

Cannabinol p-*aminobenzoate.* Cannabinol *p*-nitrobenzoate (1 g.) dissolved in alcohol (80 ml.) was hydrogenated using a platinum oxide catalyst. Absorption of hydrogen ceased when 200 ml. had been absorbed (theoretical 190 ml.). The resulting p-*aminobenzoate* crystallized from methyl alcohol in colourless needles M.P. 149–150°. (Found: C, 78·2; H, 7·4 %. $C_{28}H_{31}O_3N$ requires C, 78·3; H, 7·2 %.)

Cannabinol. Cannabinol *p*-nitrobenzoate hydrolysed by refluxing with methyl alcoholic KOH (5 %) during 1½ hr. gave cannabinol as an almost colourless oil which, with acetic anhydride-pyridine gave in quantitative yield a crystalline acetate M.P. 75° not depressed on admixture with a sample of cannabinol acetate kindly supplied by Dr R. S. Cahn.

Pharmacological tests

The Gayer test was carried out on rabbits as described by Marx & Eckhardt [1933], the substances being injected in acetone solution (0·5 % wt./vol.).

(*a*) *Cannabinol.* Pure cannabinol prepared by hydrolysing the *p*-nitrobenzoate and subsequent distillation in a high vacuum was used. In doses less than 2 mg. per kg. body weight the material had no visible effect and the corneal reflex remained normal. At any higher dosage the following sequence of events was observed. For about 1 min. the animal behaved normally, but at the end of this time it lay down and in a few seconds rolled over on its side and became rigid, the corneal reflex remaining normal. In any time from a few seconds to 1 min. later, depending on the size of the dose, the rabbit went into violent convulsions terminating in death within about 30 sec.

(*b*) *Material from non-crystalline* p-*nitrobenzoates.* After separation of the crystalline *p*-nitrobenzoate the mixture of oily esters from the resin was hydrolysed and the product distilled in a high vacuum. The nearly colourless oil obtained had no effect when injected into rabbits in doses less than 1 mg. per kg. body weight. In a dose of 1 mg. per kg. body weight the drug had no effect for about 3 min., after which time the animal's head began to nod gently and it subsided to its normal sleeping position and remained so. During this period the corneal reflex slowly disappeared until no response could be elicited. The animal could be roused for a few seconds by violent shaking but when left undisturbed quickly relapsed. In doses up to 5 mg. per kg. body weight exactly the same effect was observed, the period of sleep or stupor extending from 30 min. up to 6 or 7 hr. after which the rabbit recovered completely. In doses of 5 mg. or more the animal, although giving a positive Gayer test, died with convulsions within the first 2 hr. after injection. The death after convulsions was presumably due to the presence of some cannabinol in the injected material (cf. below).

Chromatographic analysis of hydrolysate of non-crystalline p-*nitrobenzoates*

A sample of the oil (2 g.) prepared by hydrolysis of the non-crystalline fraction of the *p*-nitrobenzoylated resin and subsequent distillation in a high vacuum was dissolved in light petroleum (300 ml.; B.P. 60–80°) and allowed to percolate through a column of activated aluminium oxide (Merck), the chromatogram being developed first with light petroleum (750 ml.; B.P. 60–80°), then with a mixture (1 litre) of equal parts of light petroleum (B.P. 60–80°) and ether.

When the column was viewed in ultraviolet light four distinct bands were visible; from the top downwards these were: (1) yellow 6 cm., (2) colourless 8 cm., (3) yellow 8 cm., (4) blue fluorescent 6 cm. On elution with a mixture of ether and methyl alcohol (4 : 1) the oils from sections (1), (2) and (3) were found to be inactive when tested on rabbits while that from section (4) gave a positive Gayer test in a dose of 3 mg. per kg. body weight.

The oil from section (4) was therefore combined with that obtained by evaporating the filtrate from the chromatogram, dissolved in light petroleum (B.P. 60–80°) containing 5 % ether and re-adsorbed on a column (2 × 30 cm.) of activated aluminium oxide (Merck), the chromatogram being developed with the same solvent mixture. From the top downwards the column showed in ultra-violet light the following bands: (1) purple 3 cm., (2) yellowish blue 10 cm., (3) deep blue 10 cm., (4) strongly blue fluorescent 2 cm., (5) yellow 3 cm. Tests on rabbits showed that the oil from section (4) (50 mg.) was lethal in a dose of 2·5 mg. per kg. body weight and had the typical action of cannabinol. The oil from section (2) (60 mg.) was active in the Gayer test in a dose of 1 mg. per kg. body weight and when injected in a dose of 5 mg. per kg. body weight the animal survived for 10 hr. without any trace of muscular rigidity and died in sleep without any convulsions. The oil from section (3), like the starting material, possessed both convulsant and sleep-producing properties.

Chromatographic analysis of non-crystalline p-*nitrobenzoates.* The oily residue (8 g.) left on removing the crystalline cannabinol derivative from the *p*-nitrobenzoylation product of the distilled resin, was dissolved in light petroleum (B.P. 40–60°) and subjected to adsorption on a column (5 × 45 cm.) of activated aluminium oxide (Merck) previously washed with a solution of phenol in light petroleum to reduce alkalinity. After developing with light petroleum (4 litres) the chromatogram showed six distinct bands when viewed in ultra-violet light. Each of these was separately eluted, hydrolysed, distilled and tested, the filtrate from the column being evaporated and the residue similarly treated and tested. The following table shows the results obtained, the bands in the chromatogram being numbered from the top downwards.

	Colour	Length cm.	Wt. of eluate g.	Millon test	Gayer test	M.L.D. mg.
1	Yellow	3	0·3	–	–	–
2	Colourless	15	1·2	–	–	–
3	Bright blue	3	0·25	+	–	6
4	Colourless	10	2·0	+	–	5·5
5	Yellow	5	0·9	–	–	–
6	Colourless	10	1·2	+	+(2 mg.)	9
7	Colourless	Filtrate	1·5	+	+(0·25 mg.)	5

The biological test results were reproducible in different animals, there being very little variation either in the minimum active dose or in the minimum lethal dose (M.L.D.). Sections (3) and (4) possessed the typical convulsion-producing properties of cannabinol while sections (6) and (7) had no such action. It is clear then from the table that the remaining traces of cannabinol were concentrated in sections (3) and (4), while the material producing corneal anaesthesia was concentrated in sections (6) and (7). The oil from sections (6) and (7) gave, like cannabinol, a precipitate with Millon's reagent.

Relative stabilities of cannabinol and resin from fraction 7 *(above).* A solution (0·5 %) of cannabinol in acetone was exposed to the air for 3 days. When tested on rabbits at the end of this time it was found to be non-toxic. A solution of the

resin from fraction 7 (above) after standing for 6 months under the same conditions retained about 25 % of its activity in the Gayer test.

SUMMARY

p-Nitrobenzoylation of the high-boiling pharmacologically active resin from the female flowers of *Cannabis indica* yields crystalline cannabinol *p*-nitrobenzoate and a mixture of resinous esters. Cannabinol is highly toxic and gives a completely negative reaction in the Gayer hashish test on rabbits, while the hydrolysis product of the resinous esters gives a strong positive reaction and is less toxic than cannabinol. The material giving a positive Gayer test has been fractionated by adsorption methods and a product obtained showing a positive Gayer test in rabbits in a dose of 0·25 mg. per kg. body weight.

One of the authors (F. B.) desires to record his appreciation of the hospitality accorded him in the University College and the Laboratory of the Public Analyst, Colombo, and to thank the Director of the Royal Botanic Gardens, Peradeniya, Ceylon, for his generous assistance.

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little depression on mixing, it was found desirable to confirm the structure of the synthetic product by degradation. When trimethylglycylcholine dibromide was shaken with silver oxide in water, it decomposed immediately, and after removal of silver salts betaine was readily isolated from the filtrate. The synthetic ester was also readily distinguished from choline by the properties of its *platinichloride*, which was very much less soluble than choline platinichloride and did not show the dimorphism characteristic of the choline salt.

Pharmacological tests on trimethylglycylcholine dibromide by Dr. F. C. MacIntosh, to whom I wish to express my thanks, showed that it possessed an extremely weak muscarine-like activity and no perceptible curare-like, nicotine-like or eserine-like activity. It was, like glycylcholine, pharmacologically rather inert.

Experimental.

β-Bromoethyl Chloroacetate.—Chloroacetyl chloride (22·6 g.) was added slowly to ethylene bromohydrin (28·0 g.) so that the temperature did not rise above 50°. The product was poured into ether, washed thoroughly with water, dried, and distilled. The fraction, b. p. 112—114°/22 mm., was collected (Found: C, 24·1; H, 3·1. $C_4H_6O_2ClBr$ requires C, 23·8; H, 3·0%); yield, 90%.

β-Chloroethyl chloroacetate, b. p. 101°/32 mm., and β-bromoethyl bromoacetate, b. p. 118°/16 mm., were prepared in the same way (cf. Henry, *Bull. Soc. chim.*, 1884, **42**, 260; Vorländer, *Annalen*, 1894, **280**, 198) in approximately the same yield.

Trimethylglycylcholine Dibromide.—β-Bromoethyl chloroacetate (20·1 g.) was sealed in a thin-walled test-tube and placed inside a Carius tube cooled in ice-salt. Cold anhydrous trimethylamine (18 c.c.) and cooled dry benzene (20 c.c.) were added, and the tube sealed as rapidly as possible; the thin inner tube was broken, and the contents thoroughly mixed. The temperature rose rapidly and crystals separated. When the initial reaction had subsided, the tube was heated at 100° for 8 hours. The solid white cake produced was broken up, washed with dry benzene, and dissolved in alcohol. A small quantity of residual insoluble material was removed and found to be betaine bromide. After several days a crystalline crust separated from the alcohol and after five recrystallisations from alcohol it was obtained in white rosettes of needles, m. p. 238°. Analysis indicated that this least soluble fraction was *trimethylglycylcholine dibromide* (Found: Br, 43·5. $C_{10}H_{24}O_3N_2Br_2$ requires Br, 44·0%). The dibromide, treated with aqueous sodium picrate, gave a *dipicrate*, which crystallised readily from aqueous alcohol in long needles, m. p. 244° (Found: C, 39·4; H, 4·5; N, 17·2. $C_{10}H_{24}O_3N_2,2C_6H_3O_7N_3$ requires C, 39·8; H, 4·5; N, 16·9%).

The alcoholic mother-liquor from the dibromide crystallisation was concentrated in the hope of obtaining the dichloride, but no crystalline material could be isolated. The yield of dibromide was about 15%. With gold chloride or platinic chloride in dilute hydrobromic acid the dibromide gave crystalline salts sparingly soluble in cold water. The gold salt crystallised in short reddish needles, m. p. about 250° (decomp.); the platinum salt crystallised in twinned prisms and melted over a range of 20° depending on the rate of heating.

A second preparation of trimethylglycylcholine dibromide carried out as described above, but using β-bromoethyl bromoacetate as starting material, gave a yield of 68% of pure dibromide.

Trimethylglycylcholine Dichloride.—β-Chloroethyl chloroacetate (7·5 g.) reacted with trimethylamine (5·63 g.) in 16 hours by the above process, but difficulty was experienced in purifying the product as the dichloride. After removal of a small quantity of betaine chloride, trimethylglycylcholine picrate was isolated, m. p. 244°, identical with the picrate obtained above.

Trimethylglycylcholine Platinichloride.—Trimethylglycylcholine chloride gave with platinic chloride a yellow crystalline precipitate sparingly soluble in cold water. The platinichloride was crystallised from hot water and from 50% aqueous alcohol, but did not show the dimorphism characteristic of choline platinichloride and crystallised from both solvents in trigonal prisms containing no water of crystallisation; these melted over a range of about 20° with variation in the rate of heating (Found: Pt, 31·2. $C_{10}H_{24}O_2N_2,PtCl_6$ requires Pt, 31·8%).

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London, N.W. 3.

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Some Aminoethanol Derivatives Possessing Local Anaesthetic Activity

By

F. C. MACINTOSH and T. S. WORK

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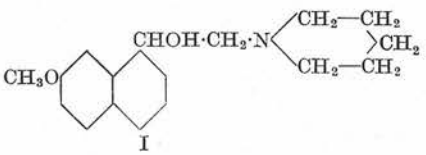
SOME AMINOETHANOL DERIVATIVES POSSESSING LOCAL ANAESTHETIC ACTIVITY

By F. C. MacIntosh and T. S. Work

(*From the National Institute for Medical Research, London*)

Received 10th December, 1940

In the course of investigations by one of us (T.S.W.) on the relation of chemical constitution to antiplasmodial action, it was noticed that piperidinomethyl-7-methoxy-1-naphthyl carbinol (I) had a strong local anaesthetic action on the tongue.



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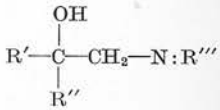


II

This substance differs in structural type from local anaesthetics hitherto described, and it seemed therefore worth while to examine a number of related compounds in the hope of finding one worthy of clinical trial. Twelve compounds were prepared and tested in greater detail. All of these have some, and several have very considerable, local anaesthetic power, but because of excessive irritant action, or for other reasons, none of them seems likely to be of practical value. As the series does not seem sufficiently promising to warrant further study at the present time, the results so far obtained are now reported.

Methods

Preparation of Compounds.—The compounds examined were all aminoethanols of the general formula (II), the substituent radicals being indicated in Table I. The general method of preparation was condensation of an α-halogenoketone with a secondary amine, followed by catalytic reduction of the resultant ketoamine, or conversion to the tertiary alcohol by means of a Grignard reagent. The syntheses of the compounds marked by an asterisk in Table I have been described elsewhere in another connection (King and Work[1], Work[2]). Details of the syntheses of the other compounds are incorporated in the present communication.

Measurement of Local Anaesthetic Activity.—The compounds were tested on the guinea-pig's cornea and on the guinea-pig's intracutaneous wheal, for their efficiency as surface anaesthetics and infiltration anaesthetics respectively. The skin of the guinea-pig is an especially useful test-object, since it can detect low grades of irritant activity, and since the animal gives clear and reliable pain signals to a light prick of the skin.

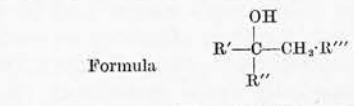
In determining the activity of a local anaesthetic one may measure either the intensity or the duration of the anaesthesia. The relative potency of two substances often depends on which of these criteria is selected. Both are important practically, but duration of anaesthesia less so than intensity, since a prolonged anaesthesia can frequently be secured by adding adrenaline to the solution. On the other hand, the intensity of a very brief anaesthesia is hard to estimate accurately. It is probably best to compromise by using an index based on both factors: in the present study we have estimated the concentration of each substance necessary to produce, by either mode of application, a full insensitivity, lasting five minutes, to a regularly repeated stimulus. This stimulus was a prick of the skin in the case of the wheal test, and the touch of a fine silk thread in the case of the cornea test.

All drugs were dissolved in 0·9 per cent. solution of sodium chloride. Each animal received at the same time, on one side the substance to be tested, and on the other procaine hydrochloride or cocaine hydrochloride. Individual variations in the effect of any one solution were considerable, but the relative effectiveness of two solutions, simultaneously administered, was reasonably constant. The potency of each substance is therefore expressed as a percentage of that of procaine hydrochloride. Each of the values given in the table is based on at least 9 such tests. The compounds were also tested for their effect on the human tongue, and some of them for their effect on the intracutaneous wheal in human skin and on the sensory and motor fibres of the frog's sciatic nerve.

Measurement of Toxicity.—LD50 for each of the compounds and for procaine and cocaine hydrochlorides was determined in mice, the drugs being given intraperitoneally dissolved in 0·9 per cent. solution of sodium chloride. At least 12 mice were used for each determination.

Measurement of Irritant Action.—The guinea-pig's skin, with intracutaneous injection, is far superior to the rabbit's cornea for the detection of low grades of irritant activity. The effect of the various compounds was roughly rated on an arbitrary scale: + being assigned to compounds producing no irritation

TABLE

Compound No.	Formula 		
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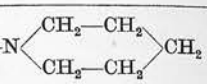
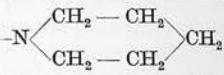
2*		H	
3*	CH_3O 	H	
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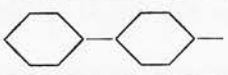
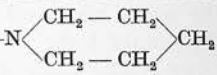
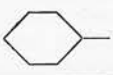
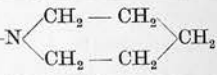
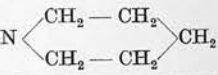
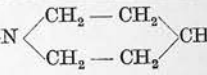
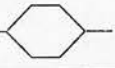
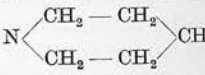
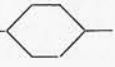
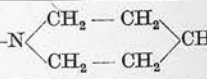
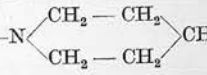
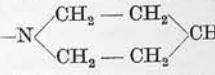
5*		H	
6		H	
7*	$C_5H_{10}N \cdot CH_2 \cdot CHOH \cdot C_6H_4$ C_6H_4	H	
8	$CH_3(CH_2)_{10}$—	H	
9	C_6H_{13}— 	H	
10	C_4H_9— 	H	
11	C_4H_9— 	C_2H_5	
12	C_4H_9— 	CH_3	
Procaine hydrochloride			
Cocaine hydrochloride			

LD50 gm./kgm.)	Anaesthetic potency (as percentage of potency of procaine hydrochloride)		action Irritant	Efficiency index	
	Guinea-pig wheal	Guinea-pig cornea		Wheal (procaine HCl=1)	Cornea (cocaine HCl=1)
200	50	100	±	0·4	0·1
250	150	500	+	1·5	0·7
175	125	300	+	0·9	0·3
175	150	400	+	1·1	0·4
175	100	5000	+++	0·7	4·7
175	25	100	+	0·2	0·1
200	75	2500	++	0·6	2·7
400	50	5000	+++++	0·8	11·0
200	50	4000	+++++	0·4	4·5
150	50	2000	+++++	0·3	1·6
200	100	2000	+++++	0·8	2·2
175	125	2000	+++++	0·8	2·0
250	100	100	—	1·0	0·15
75	250	2500	—	0·7	1·0

unless applied in concentrations of 2 per cent. or over, +++++ to compounds producing severe necrosis in concentrations of 0·2 per cent. or less, and so on.

Other Pharmacological Actions.—In a few experiments on cats anaesthetised with chloralose, the effects of the compounds on blood-pressure, pulse rate, and respiration were noted.

Results

These are summarised in Table I. The Efficiency Index of each compound is the ratio of its anaesthetic power to its toxicity: this has been calculated separately for the wheal and the cornea tests, the index for procaine hydrochloride being taken as the unit in the former case, the index for cocaine hydrochloride in the latter. The error of this index is, naturally, large, but some conclusions may be safely drawn. For infiltration anaesthesia, two compounds at least, Nos. 2 and 4, are equal or slightly superior to procaine hydrochloride, so far as local anaesthetic efficiency and toxicity are concerned. For surface anaesthesia several of the compounds, notably Nos. 8, 5 and 9 are similarly superior to cocaine hydrochloride. Unfortunately the compounds of the former group are very slightly irritant, though not appreciably so in concentrations sufficient for anaesthesia; while the compounds of the latter group are highly irritant for the guinea-pig's skin, though they abolish the corneal reflex in concentrations producing, at the most, slight irritation.

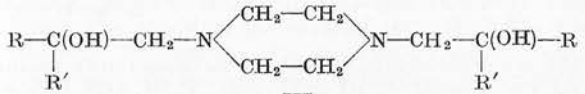
The less irritant compounds were tested on the human intracutaneous wheal, with the same results as found on guinea-pigs; and also on the frog's sciatic nerve, on which they were much less effective than procaine. The results of tests on the human tongue were intermediate between those obtained by the wheal and cornea tests, none of the compounds being, by this test, equal to cocaine hydrochloride.

The general picture of intoxication in mice was one of excitement followed by depression, excitement predominating with Nos. 5 and 6 and depression with No. 2. Given intravenously to cats in doses of 2 to 5 mgm./kgm., most of the compounds lowered the blood-pressure and slowed the pulse, with the exception of Nos. 1, 2 and 6, which produced diphasic effects, or occasionally a purely pressor and accelerator response. No. 5 differed from the other compounds in producing a prolonged bradycardia with little alteration of the arterial pressure, the force of the heart beat being augmented: this effect was not abolished by atropine or by section of the vagi, but differed in other respects from that of the cardiac glycosides. The effects of the compounds on respiration seemed to be dependent on the

circulatory changes. All the compounds considerably potentiated the pressor effect of small doses of adrenaline: it is interesting to note that they share this property, as well as local anaesthetic action, with cocaine.

Discussion

Many compounds of the general type $R'—C(OH)CH_2.N:$ (with R'' on the C) have been prepared previously (Riedel[3], Launoy[4], Fourneau[5]), but local anaesthetic activity seems to have been regarded as a property of the esters of such alcohols rather than of the alcohols themselves (*cf.* Brill and Bulow[6]). That this is not so has been noted by Fourneau and Samdahl[7]. These authors prepared a number of piperazine derivatives of the general type (III).



III

one of which was a very powerful local anaesthetic. It would appear from our results and from those of Fourneau *et al.* that local anaesthetic activity is associated with the dialkylaminoethanol structure, and is displayed whenever appropriate radicals are introduced in the positions R′ and R″. In accordance with what might be expected from the study of Adams, Rideal, Burnett, Jenkins and Dreger[8], who showed a close correlation between oil/water distribution coefficient and *surface anaesthetic activity*, the piperidinomethylcarbinol with R′ = phenyl (No. 6) was very much less active than similar compounds possessing more strongly hydrophobic groupings in the R′ position. That this difference was not dependent on molecular weight *per se* was shown by the comparative inactivity of compound No. 7. The most potent compounds were also on the whole the most irritant, probably on account of the high surface activity of their solutions. For *infiltration anaesthetic activity*, on the other hand, the naphthyl and methoxynaphthyl compounds ranked first, and the tertiary carbinols were more active than the secondary. Neither the introduction of a methoxyl group, nor the substitution of piperidine by dimethylamine, had any clear-cut effect.

Unfortunately no practical application suggests itself for any of these compounds, unless perhaps No. 8 could be used in sites, e.g. haemorrhoids, where a considerable sclerosing action is a minor disadvantage. We think it probable, however, that there may be some useful anaesthetics among aminoethanol derivatives of the types studied.

Experimental

Dimethylaminomethyl-7-methoxy-1-naphthylcarbinol.

7-Methoxy-1-naphthacylbromide (King and Work[1]) (6·0 gm.) dissolved in ether was added rapidly to a 33 per cent. solution of dimethylamine in methanol (5·7 gm.) and after two hours the mixture was diluted with ether and the precipitated dimethylamine collected (2·5 gm.). The filtrate was freed from ether without raising the temperature, made just acid to congo red by addition of hydrochloric acid and reduced catalytically using Adams' platinum oxide. The product was evaporated to dryness, dissolved in water, washed with ether, made alkaline and extracted with ether. The extracted basic oil (4·2 gm.) gave a crystalline *picrate* from methanol, m.pt. 158° C., sintering at 95°.

Found: C, 53·5; H, 4·4; N, 12·1. $C_{15}H_{19}O_2N,C_6H_3O_7N_3$ requires C, 53·2; H, 4·6; N, 11·8 per cent.

The *hydrochloride* crystallised from methanol-acetone mixture, m.pt. 209° C. Found: C, 64·1; H, 6·8. $C_{15}H_{19}O_2N,HCl$ requires C, 63·9; H, 7·1 per cent.

Piperidinomethyl-1-phenylcarbinol.

The method was the same as in the preceding experiment. Bromoacetophenone (6·0 gm.) condensed with piperidine (5·5 gm.) in ether to give piperidine hydrobromide (4·8 gm.) and a base which absorbed 700 mils of hydrogen on reduction. The product was isolated as the *hydrochloride*, soluble in methanol, sparingly soluble in acetone, m.pt. 195° C.

Found: C, 64·6; H, 8·2; N, 5·6. $C_{13}H_{19}ON,HCl$ requires C, 64·6; H, 8·3; N, 5·8 per cent.

Hexylbenzene.

Hexylbenzene was prepared from caproyl benzene by two methods. Caproyl benzene (15 gm.) was converted to the semicarbazide, m.pt. 134° and this was heated for eighteen hours in a sealed tube with sodium ethoxide (2·3 gm. of sodium in 30 mils of alcohol): hexylbenzene, b.pt. 101 to 103° C./12 mm., was obtained by fractional distillation of the product. A second portion of caproyl benzene (55·0 gm.) was reduced by treatment with zinc amalgam (200 gm.) in a mixture of concentrated hydrochloric acid and alcoholic hydrochloric acid as described by Harris, Marriot and Smith[9] in their preparation of heptylbenzene. The yields were much the same in both these methods (*ca.* 50 per cent.) but the second was found the more convenient.

p-Hexyl-ω-chloracetophenone.

Hexylbenzene (23 gm.) and chloracetyl chloride (16 gm.) were mixed in dry carbon disulphide (50 c.c.) and powdered aluminium chloride (23 gm.) was added. The reaction was complete in one hour and the product was then decomposed with acid and the *hexylchloracetophenone* extracted with petroleum, dried and distilled, b.pt. 154° to 156° C./0·9 mm., m.pt. 32° C., yield 22·0 gm.

Found: C, 70·0; H, 7·9. $C_{14}H_{19}OCl$ requires C, 70·4; H, 8·0 per cent.

Piperidinomethyl-1-(p-hexylphenyl) carbinol.

p-Hexyl-ω-chloracetophenone (18·0 gm.) in ether (25 mils) was condensed with piperidine (12·8 gm.) in ether, the mixture being warmed to 35° C. for fifteen minutes at the end of two hours to complete the reaction. Piperidine hydrochloride (8·1 gm.) was collected. The ketone was reduced in the normal way in acid alcohol and the desired base isolated as the picrate, m.pt. 133° to 135° C. from methanol, yield 70 per cent.

Found: C, 57·9; H, 6·8; N, 10·8. $C_{19}H_{31}ON,C_6H_3O_7N_3$ requires C, 57·9; H, 6·6; N, 10·8 per cent.

Butylbenzene.

Butylbenzene was prepared by the modified Clemmensen reduction already mentioned; the yield was about 50 per cent.

p-Butyl-ω-chloracetophenone.

Butylbenzene (7·0 gm.) and chloracetylchloride (5·9 gm.) in carbon disulphide were condensed with the aid of aluminium chloride (9·0 gm.) and the desired *ketone* (6·6 gm.), b.pt. 142° to 144° C./2·0 mm., obtained by the same procedure as in the preparation of the higher homologue.

Found: C, 68·2; H, 6·9. $C_{12}H_{15}OCl$ requires C, 68·4; H, 7·1 per cent.

Piperidinomethyl-1-(p-butylphenyl) carbinol.

Butyl-ω-chloracetophenone (6·6 gm.) and piperidine (5.35 gm.) condensed as described already for the higher homologue gave an oily aminoketone (8·0 gm.). This oil (2·5 gm.) reduced catalytically gave the desired aminoalcohol (2·4 gm.), isolated as the crystalline *picrate*, m.pt. 137° to 138° C., from ethanol.

Found: C, 56·9; H, 5·7. $C_{17}H_{27}ON,C_6H_3O_7N_3$ requires C 56·4; H, 6·1 per cent.

Ethyl-piperidinomethyl-1-(*p-butylphenyl*) *carbinol.*

p-Butyl-ω-piperidinoacetophenone (2·5 gm.) in dry ether was added slowly to ethyl magnesium iodide (2 mols) cooled in ice. After twelve hours the mixture was poured on to a mixture of ice and hydrochloric acid, washed with ether, made alkaline and the free base extracted with ether. The base was partially purified by re-extraction from ether with acid followed by liberation of the base with alkali. The final oily product was dissolved in dry alcoholic hydrochloric acid, acetone and ether were added very slowly and the crystalline *hydrochloride* was collected. After recrystallisation from acetone the product melted at 178° C., yield 0·6 gm.

Found: C, 70·5; H, 9·9. $C_{19}H_{31}ON,HCl$ requires C, 70·0; H, 10·0 per cent.

Methyl piperidinomethyl-1-(*p-butylphenyl*) *carbinol.*

In view of the poor yield obtained in the above experiment, this preparation was modified so that the Grignard reagent and chloroketone were condensed before combination with piperidine. Butyl-ω-chloracetophenone (6·3 gm.) in ether (25 c.c.) was added slowly to an ice-cooled ether solution of methyl magnesium iodide (1 mol. magnesium, 0·72 gm.). After two hours the mixture was treated with ice and sulphuric acid and the product extracted with ether, washed and dried. The resulting brown oil (6·35 gm.) solidified partially on standing and a white crystalline substance (2·2 gm.), m.pt. 121° C., was isolated from it and appeared to have the empirical formula $C_{12}H_{16}O$.

Found: C, 82·1; H, 8·6. $C_{12}H_{16}O$ requires C, 81·8; H, 9·1 per cent.

It was not further examined. The remaining oily fraction was warmed on the water-bath with excess of piperidine for two minutes, cooled, and poured into acid. Unchanged material was recovered by extraction of the acid solution with ether. By this process unchanged α-chloroketone in which the chlorine is much more highly reactive than in the desired tertiary alcohol was removed as acid soluble aminoketone. The purified oil (3·5 gm.) was refluxed with excess of piperidine for twelve hours, poured into acid and washed with ether, made alkaline, extracted with ether and the ether washed very thoroughly with water until free from piperidine. The yellow oil obtained from the ether gave a crystalline *hydrochloride*, m.pt. 186° C., from acetone, yield 0·8 gm.

Found: C, 68·8; H, 9·3; N, 4·4. $C_{18}H_{29}ON,HCl$ requires C, 68·8; H, 9·7; N, 4·5 per cent.

1-*Chlorotridecanone*-2.

Lauric acid chloride (11·0 gm.) in ether was added slowly to an ether solution of diazomethane (from 20 gm. nitrosomethylurea). After twelve hours the flocculent precipitate was removed and the ether evaporated. The pale yellow diazoketone was moderately soluble in petroleum, m.pt. 44° C. The diazoketone dissolved in ether was decomposed by dry hydrogen chloride, the ether evaporated and the product crystallised from petrol, m.pt. 46° C., yield 10·1 gm.

Found: C, 67·3; H, 10·3. $C_{13}H_{25}OCl$ requires C, 67·1; H, 10·17 per cent.

Piperidinomethyl-undecylcarbinol.

Chlorotridecanone (7·3 gm.) in ether was condensed with piperidine (5·5 gm.) as in previous analogous experiments. Piperidine hydrochloride (3·25 gm.) was removed and the aminoketone reduced in the usual way. Reduction was somewhat more troublesome than with the other compounds but was completed by using a large quantity of fresh catalyst. (*Cf.* Work, *loc. cit.*). The piperidinomethyl-undecylcarbinol (7·02 gm.) was isolated as the *picrate*, m.pt. 69° to 70°.

Found: C, 56·6; H, 17·5. $C_{18}H_{37}ON,C_6H_3O_7N_3$ requires C, 56·2; H, 17·8 per cent.

Summary.

Twelve aminoethanol derivatives in which the hydroxyl group remains unesterified have been prepared. Some of these are very potent local anaesthetics, but are too irritant to be of practical value.

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BIOCHEMISTRY

6. THE BIOCHEMICAL EFFECTS OF MUTATION

BY
T. S. WORK

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6. The Biochemical Effects of Mutation.

To the biochemist cell metabolism is essentially a series of integrated enzymic reactions, to the geneticist the cell is a self-reproducing unit controlled by genes. Interest in the effect of gene mutation on metabolism was at first confined to the study of *inborn errors of metabolism* in man,[1] but has gradually extended to other animals,[2] to plants,[3] to insects,[2, 4] to micro-organisms,[5] and to viruses.[6]

When mutation results in the excretion of an unusual metabolite the difficulty always exists of assessing the significance of that metabolite as an intermediate of normal metabolism. A. Neuberger, C. Rimington, and J. M. G. Wilson[7] suggest from a detailed study of alcaptonuria that oxidation of phenylalanine and tyrosine to homogentisic acid represents the main, but probably not the only, catabolic pathway for these two amino-acids both in the alcaptonuric and in the normal individual.

Study of anthocyanin synthesis[3, 8] in flowers suggested that chemical reactions were under the control of individual genes. Interpretation of mutation in terms of biochemistry in *Drosophila melanogaster* has proved considerably more difficult, despite rapid progress in relating phenotype character to chromosome structure in mutant flies. Failure to breed Drosophila on a pure synthetic medium has been one limiting factor. Considerable progress has now been made towards definition of the nutritional

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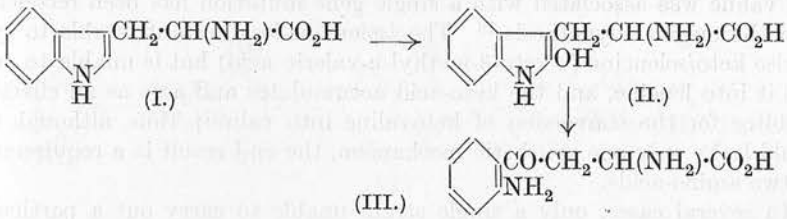
requirements of insect larvæ.[9] The so-called v^+ hormone which produces brown pigment in Drosophila eyes and also in the eyes of the flour moth, *Ephestia kuhniella*, has been identified as kynurenine, a product of tryptophan metabolism.[10] Kynurenine has the structure (III) and is probably formed from tryptophan (I) through the intermediate (II).[11]



Apparently brown eye and white eye strains differ with respect to genes controlling kynurenine synthesis; thus, a white eye strain of Ephestia *aa* is homozygous with respect to gene *a* which causes lack of kynurenine. It was claimed [12] that oxidation of tryptophan to α-hydroxytryptophan was the missing step in an *aa* strain, since injection of α-hydroxytryptophan caused pigment formation, but E. Caspari [13] found that a tissue homogenate of *aa* Ephestia larvæ oxidised tryptophan as readily as a similar extract from $a^+ a^+$ larvæ. In both cases the product had the character of an ommochrome pigment.[14] Recent results of E. Caspari [15, 13] suggest that in *aa* strains conversion of tryptophan into kynurenine takes place at a reduced rate so that kynurenine does not accumulate. This result is reflected in the abnormally high tryptophan content of protein from an *aa* strain. Kynurenine-deficient Drosophila did not show a similar high tryptophan content.[15]

Mutation in Moulds.—The introduction of methods for isolation and genetic analysis of radiation induced mutants of Neurospora has led since 1941 to a sudden blossoming of biochemical genetics.[16] Wild type Neurospora requires, for growth, a source of organic carbon, such as glucose, a source of ammonium ion, inorganic salts, and one specialised molecule, biotin. Systematic study of induced mutants has led to the isolation of strains with requirements for each of the B vitamins except folic acid and of other strains requiring one or other of a dozen amino-acids.[17] Inability to carry

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*

out the synthesis of an essential metabolite is inherited as though differentiated from normal by a single gene, and the working hypothesis has been proposed that each step in a series of linked biochemical reactions is controlled by a single gene.

One apparent exception in which a double requirement for *iso*leucine and valine was associated with a single gene mutation has been reconciled with the original hypothesis.[18] The *iso*leucine-less mutant is able to synthesise keto*iso*leucine (α-keto-β-methyl-*n*-valeric acid) but is unable to convert it into leucine, and the keto-acid accumulates and acts as an effective inhibitor for the conversion of ketovaline into valine; thus, although the mould lacks only one synthetic mechanism, the end result is a requirement for two amino-acids.

In several cases, only a single strain unable to carry out a particular biosynthesis has been isolated, but for several metabolite deficiencies a number of genetically distinct strains have been obtained, each apparently unable to carry out a single step in a metabolic chain of reactions leading to a single essential metabolite. Such genotypically different strains requiring the same growth factor may furnish valuable evidence for the consecutive steps of a biosynthetic pathway.

Induced mutants of Neurospora have been isolated which require nicotinic acid or nicotinamide for growth. Genetic analysis indicated that nicotinic acid synthesis might be blocked at at least three separate loci. Pyridine, β-picoline, γ-picoline, piperidine, piperidine-3-carboxylic acid, trigonelline, ornithine, proline, α-amino-*n*-valeric acid, and α-amino-*n*-hexoic acid, possible precursors of nicotinic acid, were all unable to replace nicotinic acid for growth of any of the mutants and are, therefore, not likely intermediates in nicotinic acid synthesis.[19] The observation that in growing rats tryptophan can replace nicotinic acid [20] and that the two compounds are interchangeable in counteracting the pellagra-like effects of 3-acetylpyridine [21] suggested that tryptophan might be a precursor of nicotinic acid. Two nicotinic acid-less mutant strains of Neurospora (A) and (B) differing from the normal by a single gene have been found to be able to convert tryptophan into nicotinic acid. Both these strains utilised kynurenine as well as tryptophan.[22] Two other genetically distinct nicotinic acid-less strains (C) and (D) failed to use kynurenine or tryptophan. When supplied with minimal quantities of nicotinic acid, strain C produced, in the medium, two growth factors which could replace nicotinic acid in the nutrition of strain B. These growth factors were isolated in crystalline form.[19] Elementary analysis, equivalent-weight determinations, and chemical properties suggest that the two com-

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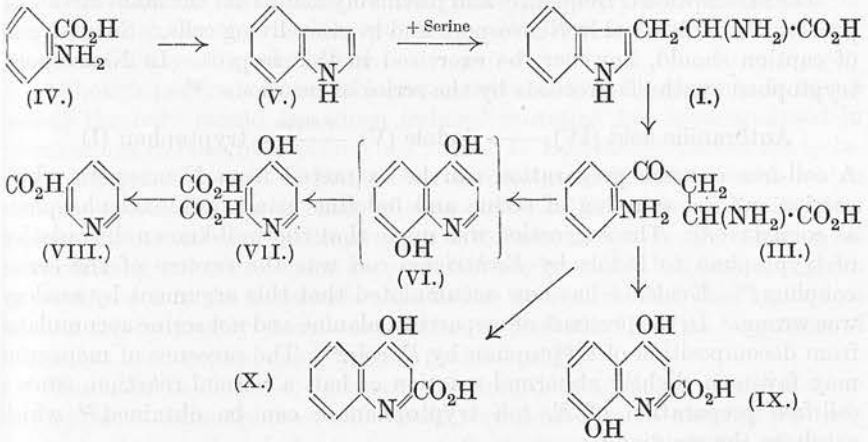
[21] D. W. Woolley, *J. Biol. Chem.*, 1946, **162**, 179.

[22] G. W. Beadle, H. K. Mitchell, and J. F. Nye, *Proc. Nat. Acad. Sci.*, 1947, **33**, 155.

pounds are closely related heterocyclic carboxylic acids, possibly hydroxypyridinecarboxylic acids.*



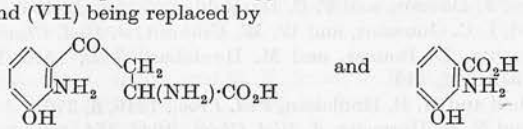
If it is accepted that tryptophan (I) and kynurenine (III) are precursors of nicotinic acid (VIII), then some intermediate quinoline derivative such as (VI) must be postulated in order to produce a pyridine-3-carboxylic acid (VII) which could be converted into nicotinic acid. According to this scheme mutants (C) and (D) would lack enzymes necessary for the conversion of (VII) into (VIII) and might be expected to accumulate hydroxypyridinecarboxylic acids when grown in minimal concentrations of nicotinic acid. Quinolinecarboxylic acids of type (VI) might be formed from kynurenine since animals reared on a pyridoxin-deficient diet excrete xanthurenic acid (IX).[23] Transformation of tryptophan through kynurenine to nicotinic acid cannot, however, be accepted at present as a general mechanism. A number of workers [24] have shown that, in animals, oral administration of tryptophan gives increased urinary excretion of nicotinic acid derivatives but F. Rosen, J. W. Huff, and W. A. Perlzweig [25] have failed to find any increase

[23] S. Lepkovsky, E. Roboz, and A. J. Haagen-Smit, *J. Biol. Chem.*, 1943, **149**, 195.

[24] F. Rosen, J. W. Huff, and W. A. Perlzweig, *ibid.*, 1946, **163**, 343; B. S. Schweigert and P. B. Pearson, *ibid.*, 1947, **168**, 555; W. A. Perlzweig, F. Rosen, N. Levitas, and J. Robinson, *ibid.*, 1947, **167**, 511.

[25] *J. Nutrition*, 1947, **33**, 561.

* *Note added in Proof.*—One of these compounds has now been identified as 3-hydroxyanthranilic acid (H. K. Mitchell and J. F. Nye, *Proc. Nat. Acad. Sci.*, 1948, **34**, 1; D. Bonner, *ibid.*, p. 5). The suggested reaction scheme may therefore be modified, (VI) and (VII) being replaced by



respectively. Several hydroxypyridinecarboxylic acids were synthesised and found to be inactive in promoting growth of the test strain.

in urinary *N*-methylnicotinamide after administration of kynurenine (III), kynurenic acid (X), or xanthurenic acid (IX).

The assumption is frequently and justifiably made that the main metabolic pathways are identical in Neurospora and in other living cells. Some degree of caution should, however, be exercised in this respect. In Neurospora, tryptophan synthesis proceeds by the series of reactions : [26]

$$\text{Anthranilic acid (IV)} \longrightarrow \text{indole (V)} \xrightarrow{+\text{Serine}} \text{tryptophan (I)}$$

A cell-free enzyme preparation can be extracted from Neurospora which carries out the coupling of serine and indoline using pyridoxal phosphate as coenzyme.[27] The suggestion was made that the well-known degradation of tryptophan to indole by *Escherichia coli* was the reverse of the serine coupling.[26] Evidence has now accumulated that this argument by analogy was wrong. In the presence of mepacrine, alanine and not serine accumulates from decomposition of tryptophan by *E. coli*.[28] The presence of mepacrine may favour a slightly abnormal reaction or halt a normal reaction, since a cell-free preparation of *E. coli* tryptophanase can be obtained [29] which catalyses the reaction :

$$\text{Tryptophan} \longrightarrow \text{indole} + \text{pyruvic acid} + \text{ammonia.}$$

The enzyme can be resolved into an inactive apo-enzyme and a coenzyme. The apo-enzyme was fully reactivated by pyridoxal phosphate. The purified enzyme does not deaminate serine or alanine; thus, neither of these amino-acids is an intermediate.

Induced mutants of Neurospora have provided fresh evidence for the following biosynthetic pathways :

(*a*) aminoethanol $\longrightarrow$ methylaminoethanol $\longrightarrow$ dimethylaminoethanol $\downarrow$ choline [30]

(*b*) hypoxanthine $\longrightarrow$ adenine [31]

(*c*) ornithine $\longrightarrow$ citrulline $\longrightarrow$ arginine [32]

(*d*) cysteine $\longrightarrow$ cystathionine $\longrightarrow$ homocystine $\longrightarrow$ methionine [33]

It should be remarked in connection with arginine-less Neurospora that observation of a series of gene-controlled reactions involving ornithine, citrulline, and arginine only provides evidence for the route of arginine synthesis and does not, as has been suggested,[34] provide evidence in favour of

[26] E. L. Tatum and D. Bonner, *Proc. Nat. Acad. Sci.*, 1944, **30**, 30; E. L. Tatum, D. Bonner, and G. W. Beadle, *Arch. Biochem.*, 1944, **3**, 477.

[27] W. W. Umbreit, W. A. Wood, and I. C. Gunsalus, *J. Biol. Chem.*, 1946, **165**, 731.

[28] E. A. Dawes, J. Dawson, and F. C. Happold, *Biochem. J.*, 1947, **41**, 426.

[29] W. A. Wood, I. C. Gunsalus, and W. W. Umbreit, *J. Biol. Chem.*, 1947, **170**, 313.

[30] N. H. Horowitz, D. Bonner, and M. Houlahan, *ibid.*, 1945, **159**, 145; N. H. Horowitz, *ibid.*, 1946, **162**, 413.

[31] H. K. Mitchell and M. B. Houlahan, *Fed. Proc.*, 1946, **5**, 370.

[32] A. M. Srb and N. H. Horowitz, *J. Biol. Chem.*, 1944, **154**, 129.

[33] N. H. Horowitz, *ibid.*, 1947, **171**, 255.

[34] G. W. Beadle, *Physiol. Rev.*, 1945, **25**, 643.

the Krebs–Henseleit cycle. Recent evidence suggests that this cycle as originally proposed is not necessarily either the only or the main pathway for urea synthesis in animals.[35] The presence of arginase and urease in Neurospora shows that such a cycle is possible, but it does not necessarily show that it actually takes place.

Although particularly suitable for genetic studies, Neurospora is by no means the only mould for which induced mutation has been analysed in terms of loss of biosynthetic enzymes; thus, D. Bonner [36] reported the isolation of mutant strains of *Penicillium notatum* and *P. chrysogenum* which failed to synthesise one or other of nine amino-acids and seven B vitamins. Induced mutants of penicillium have also been useful in the selection of commercially valuable strains for penicillin production,[37] but have not given any indication of the biogenesis of penicillin beyond the fact that lysine-less strains are frequently unable to synthesise penicillin.[38] The biogenesis of biotin has been studied using mutant penicillium.[39]

Mutations in Bacteria.—Absence of recognisable nuclear division or of any apparent sexual phase which would permit analysis by classical methods has retarded study of the genetic and biochemical character of strain variation in bacteria. Any attempt at analysis must be made, not in the individual cell, but upon a cell population, so that to the plasticity of the individual must be added the plasticity and possible heterogeneity of the population as a whole; nevertheless, the biochemical versatility and speed of reproduction of bacteria make them in some respects almost ideal material for a study of the biochemistry of variation.

It is a characteristic of gene mutation that it occurs spontaneously with a definite frequency and is fairly permanent. Various physical and chemical agencies can be used to increase the frequency of mutation in genetically defined organisms. The same agencies produce strain variation in bacteria; thus, exposure of bacteria to *X*-rays [40, 41] produces new strains showing specific growth-factor deficiencies analogous to those reported in Neurospora. Un-irradiated strains produce similar variants with a lower frequency than irradiated cultures.[41] Re-exposure of a nutritionally deficient strain to *X*-rays may produce cells with multiple deficiencies. Exposure to ultra-violet light, another mutation-producing agency, also induces specific growth-factor deficiency in bacteria,[42] and increases the frequency of occurrence of phage resistance in *Escherichia coli*.[43]

[35] P. P. Cohen and M. Hayano, *J. Biol. Chem.*, 1947, **170**, 687; S. Ratner, *ibid.*, p. 761; H. Borsook and J. W. Dubnoff, *ibid.*, 1947, **169**, 461.

[36] *Amer. J. Bot.*, 1946, **33**, 788.

[37] M. P. Backus, J. F. Stauffer, and M. J. Johnson, *J. Amer. Chem. Soc.*, 1946, **68**, 152.

[38] D. Bonner, *Arch. Biochem.*, 1947, **13**, 1.

[39] *J. Biol. Chem.*, 1945, **160**, 455.

[40] R. R. Roepke, R. L. Libby, and M. H. Small, *J. Bact.*, 1944, **48**, 401; C. H. Gray and E. L. Tatum, *Proc. Nat. Acad. Sci.*, 1944, **30**, 404.

[41] E. L. Tatum, *ibid.*, 1945, **31**, 215.

[42] J. S. Lederberg, quoted by E. L. Tatum, *Cold Spring Harbor Symp.*, 1946, **11**, 278.

[43] M. Demerec, *Proc. Nat. Acad. Sci.*, 1946, **32**, 36.

Chemical induction of mutation in Drosophila and in Neurospora by 2-chloroethyl sulphides or 2-chloroethylamines has been conclusively demonstrated.[44, 45] Exposure of *Penicillium notatum* to methyldi-2-chlorethylamine also gave rise to mutant strains.[46] Di-2-chlorethylamine has been found to be as effective as *X*-rays in producing biochemically altered strains of *Escherichia coli*.[47] This work was facilitated by the introduction of an ingenious new method for the isolation of biochemically deficient variants.[48]

Synthetic deficiencies induced in bacteria by exposure to radiation or to 2-chlorethyl sulphides or 2-chloroethylamines were exactly similar to those known to be associated with gene mutation in higher organisms. It would, therefore, appear to be a reasonable working hypothesis that bacteria, like higher organisms, possess a gene-like mechanism for the control of heredity and of individual biochemical reactions. This view is considerably strengthened by the important work of J. Lederberg and E. L. Tatum [49] on gene recombination in *E. coli*. Genetically marked strains of *E. coli* were obtained by multiple induced mutation. These strains, when kept in pure culture, bred true and did not revert to the " wild " type, but, when two strains were mixed and cultured together, new strains arose which had regained the ability to live on the simplest media and were biochemically indistinguishable from the original " wild " strain. Ultra-violet spectrography and Feuglen staining technique also suggest that some bacteria possess a nuclear mechanism which is similar in function, if not in morphology, to that of metazoan cells.[50]

Although the biochemical conversion of phenylalanine into tyrosine has been demonstrated in animals, S. Simmonds, E. L. Tatum, and J. S. Fruton [51] suggest from a study of the nutritional requirements of phenylalanine-less and tyrosine-less mutants of *Escherichia coli* that conversion of phenylalanine into tyrosine does not occur in this organism; a conclusion at variance with other work based on inhibition studies with β-2-thienylalanine.[52] Phenylalanine-less *E. coli* was able to use simple peptides of phenylalanine for growth but was unable to use dehydrophenylalanine or acetyldehydrophenylalanine.[51]

Arginine-less mutants of *E. coli* analogous to arginine-less Neurospora

[44] C. Auerbach and J. M. Robson, *Nature*, 1946, **157**, 302; C. Auerbach, J. M. Robson, and J. G. Carr, *Science*, 1947, **105**, 243; C. Auerbach, *Genetics*, 1947, **32**, 3; cf. also A. Gilman and F. S. Philips, *Science*, 1946, **103**, 409.

[45] N. H. Horowitz, M. B. Houlahan, M. G. Hungate, and B. Wright, *ibid.*, 1946, **104**, 233.

[46] M. A. Stahmann and J. F. Stauffer, *ibid.*, 1947, **106**, 35.

[47] E. L. Tatum, *Cold Spring Harbor Symp.*, 1946, **11**, 278; S. Simmonds, E. L. Tatum, and J. S. Fruton, *J. Biol. Chem.*, 1947, **169**, 91.

[48] J. Lederberg and E. L. Tatum, *ibid.*, 1946, **165**, 381.

[49] *Nature*, 1946, **158**, 558; E. L. Tatum and J. Lederberg, *J. Bact.*, 1947, **53**, 673; J. Lederberg, *Genetics*, 1947, **32**, 505.

[50] B. Malmgren and G. G. Hedén, *Nature*, 1947, **159**, 578.

[51] *J. Biol. Chem.*, 1947, **169**, 91.

[52] E. Beerstecher and W. Shive, *ibid.*, 1947, **167**, 49.

substantiate the biosynthetic sequence proposed for arginine.[53] Other induced mutants of *E. coli* provide evidence for the occurrence of the following biosynthetic reactions : anthranilic acid ⟶ indole ⟶ tryptophan; glutamic acid ⟶ proline; serine ⟶ glycine; vitamin B_1-thiazole ⟶ thiamin; adenine ⟶ guanine.[53, 55] A particularly complete series of biochemical mutants of *E. coli* concerned with sulphur metabolism indicate the following sequence :

$$SO_4 \longrightarrow SO_3^{=} \longrightarrow S^{=} \longrightarrow \text{cysteine} \longrightarrow [\text{cystathionine}] \longrightarrow \text{homocysteine} \longrightarrow \text{methionine.}^{54}$$

A novel type of mutation has been reported in *Moraxella lwoffi*. The normal strain cannot grow on succinate, fumarate, malate, or oxalacetate, but it produces a mutant which can. Metabolism of acetate or lactate by the normal strain is not inhibited by the four dicarboxylic acids. A. Lwoff and A. Audureau [56] suggest that the mutant utilises dicarboxylic acids by an abnormal metabolic route involving phosphorylative degradation of malate or possibly oxalacetate to pyruvate. Mutation occurs spontaneously but the mutation rate can be increased by exposure to *X*-rays.[57]

Instability of biochemical and morphological character has been regarded as an argument against the mutational nature of bacterial variation,[58] but when it is remembered that a single organism may give rise in less than 24 hours to more than 10^{10} descendants this instability is not surprising. Spontaneous mutation in genetically defined micro-organisms is by no means rare.[59]

Spontaneous reverse mutation from leucine-less to leucine-independent has been demonstrated in Neurospora. As might be expected on theoretical grounds, a double mutant with two combined nutritional deficiencies reverted to a non-exacting form with almost zero frequency.[60] It is particularly significant that, in the presence of sufficient leucine, the leucine-less strain had a growth advantage over its leucine-independent mutant. A methionine-less mutant of *Escherichia coli* has also been reported with a growth rate greater than the non-deficient strain.[61] If an increased growth rate in a rich medium can be acquired by loss of synthetic function, the poor synthetic ability of highly parasitic micro-organisms becomes at once understandable. Conversely, " training " of a nutritionally-exacting

[53] R. R. Roepke, *J. Bact.*, 1946, **52**, 504; cf. E. L. Tatum, *Cold Spring Harbor Symp.*, 1946, **11**, 278.

[54] J. O. Lampen, R. R. Roepke, and M. J. Jones, *Arch. Biochem.*, 1947, **13**, 55; A. Lwoff, *Proc. Soc. Gen. Microbiol., Oxford*, Sept. 1947.

[55] E. L. Tatum, *Proc. Nat. Acad. Sci.*, 1945, **31**, 215; *Cold Spring Harbor Symp.*, 1946, **11**, 278.

[56] *Ann. Inst. Pasteur*, 1947, **73**, 517.

[57] R. Croland, *Compt. rend.*, 1943, **216**, 616.

[58] W. Braun, *Bact. Rev.*, 1947, **11**, 75.

[59] C. C. Lindegren, *ibid.*, 1945, **9**, 111.

[60] F. J. Ryan, *Cold Spring Harbor Symp.*, 1946, **11**, 215.

[61] J. Monod, *Ann. Inst. Pasteur*, 1946, **72**, 879.

pathogenic strain to be independent of a particular nutrient can probably be regarded as a process of selection of spontaneously-occurring back utants;[61, 62] however, a true chemical guidance of mutation is by no means excluded.[75, 76] Spontaneous back mutation in *Escherichia coli* from histidine dependence to histidine independence has been reported to occur with a frequency of 10^{-8} per cell per generation.[60]

The now classical analysis of bacteriophage-resistant mutation in *Escherichia coli* initiated by S. E. Luria and M. Delbruick [63] has been followed by application of similar methods to studies of the origin of drug resistance. The conclusion has been reached that, in many cases, drug resistance develops by a mechanism akin to mutation and is independent of the presence of the drug which acts solely by a selective mechanism.[64] It is by no means certain, however, that all examples of development of drug resistance are of this type.[65] A biochemical basis for bacteriophage resistance in *Escherichia coli* has been indicated by the observations of E. H. Anderson,[66] T. F. Anderson,[67] and E. Wollman [68] that resistance is associated with loss of ability to synthesise tryptophan or proline. The specificity requirement of the phage for tryptophan is not absolute and other β-aryl-α-amino-acids can replace it to some extent in promoting phage attack.[67]

Mutations induced by radiation or by 2-chloroethyl sulphides or 2-chloroethylamines are random, but the type transformation of pneumococcus induced by a nucleic acid extract of the S-form of another type has the character of an induced non-random mutation.[69] This is not an isolated and exceptional case since similar type transformation has been reported in *Escherichia coli*.[70] In each case transformation involved acquisition by the organism of new synthetic abilities. S. Spiegelman suggests that the enzymic constitution of one strain of yeast may be influenced by a nucleoprotein extract of another strain.[71]

There is no *a priori* reason to assume that mutation cannot be guided as well as induced by suitable chemicals. The carcinogenic hydrocarbons

[62] S. E. Luria, *Bact. Rev.*, 1947, **11**, 1.

[63] *Genetics*, 1943, **28**, 491; M. Demerec and U. Fano, *ibid.*, 1945, **30**, 119.

[64] M. Demerec, *Proc. Nat. Acad. Sci.*, 1945, **31**, 16; *Ann. Missouri Bot. Gdn.*, 1945, **32**, 131; E. Oakberg and S. E. Luria, *J. Bact.*, 1946, **52**, 152; M. Klein and L. J. Kimmelman, *ibid.*, p. 471; M. Klein, *ibid.*, 1947, **53**, 463; C. A. Chandler and E. B. Schoenbach, *Proc. Soc. Exp. Biol. Med.*, 1947, **64**, 208; H. E. Alexander and G. Leidy, *J. Exp. Med.*, 1947, **85**, 329.

[65] C. N. Hinshelwood, "The Chemical Kinetics of the Bacterial Cell," Oxford, 1946.

[66] *Proc. Nat. Acad. Sci.*, 1944, **30**, 397; 1946, **32**, 120.

[67] *Cold Spring Harbor Symp.*, 1946, **11**, 1.

[68] *Ann. Inst. Pasteur*, 1947, **73**, 348.

[69] M. McCarty, *Bact. Rev.*, 1946, **10**, 63.

[70] A. Boivin, A. Delaunay, R. Vendrely, and Y. Lehoult, *Compt. rend.*, 1945, **221**, 718; A. Boivin, R. Vendrely, and Y. Lehoult, *ibid.*, p. 646; R. Vendrely and Y. Lehoult, *ibid.*, 1946, **222**, 1357; A. Boivin, A. Delaunay, R. Vendrely, and Y. Lehoult, *Experientia*, 1946, **2**, 139.

[71] *Cold Spring Harbor Symp.*, 1946, **11**, 256.

induce mutation in Drosophila,[72] in mice,[73] and possibly in bacteria.[74] Dibenzanthracene-induced mutation in the mouse may be a guided rather than a random mutation.[75] On the basis of guided mutation chemotherapeutic control of cancer seems at least a distinct possibility.

T. S. W.

D. J. Bell.
E. G. Hughes.
A. Lawson.
C. Rimington.
R. Scarisbrick.
T. S. Work.

[72] M. Demerec, *Nature*, 1947, **159**, 604.
[73] L. C. Strong, *Proc. Nat. Acad. Sci.*, 1945, **31**, 290; J. G. Carr, *Brit. J. Cancer*, 1947, **1**, 152.
[74] P. A. Ark, *J. Bact.*, 1946, **51**, 699.
[75] J. G. Carr, *Brit. J. Cancer*, 1947, **1**, 152.
[76] C. C. Lindegren and C. Raut, *Ann. Missouri Bot. Gdn.*, 1947, **34**, 85.

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Bungay, Suffolk.

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ISOLATION OF A CRYSTALLINE TOXIC FACTOR FROM AGENIZED WHEAT FLOUR

By Dr. P. N. CAMPBELL, Dr. T. S. WORK and Sir EDWARD MELLANBY, G.B.E., K.C.B., F.R.S.

National Institute for Medical Research and Nutrition Building, Mill Hill, London, N.W.7

IN 1946 it was established by Mellanby[1] that dogs fed a diet rich in commercially 'improved' (nitrogen trichloride-treated) flour were subject to epileptiform fits; similar fits were never seen in control animals fed on untreated flour. Mellanby concluded that the treatment of flour with nitrogen trichloride gave rise to a toxic factor which was responsible for the condition in dogs well known in Great Britain as 'canine hysteria' and in the United States as 'running fits'.

A large proportion of the flour milled in Britain and used for bread-making is 'improved' by treatment with nitrogen trichloride and is known commercially as 'agenized' flour. The observation that this flour was toxic to dogs was followed by a demonstration that it was also toxic to ferrets[2], to cats[3], and to rabbits[4]. It thus became a matter of some importance to discover the nature of the toxic factor and to assess the possible danger which might arise from the use of agenized flour.

Method of Test

Throughout the present investigation the criterion of toxicity adopted has been the production of a typical epileptiform fit in the ferret. The ferret is slightly less sensitive than the dog on a body-weight basis; but the small size of the animal enabled us to obtain a toxic reaction with a much smaller dose of agenized flour than would be necessary for a dog.

The demonstration by Mellanby[2] that toxicity was confined to the gluten fraction of the flour suggested that the toxic factor might be a modified amino-acid or peptide. The final method of isolation which is outlined below was based on this assumption.

Isolation of Crystalline Toxic Factor

Flour was agenized by the standard procedure but with ten times the amount of nitrogen trichloride used in commercial practice (11·1 gm. NCl_3 to 17·7 kgm. flour). This flour was toxic to a ferret in

a dose of 100 gm. (1·9 gm. nitrogen) fed over three days. Gluten was separated from the flour and digested first with pepsin and then with trypsin. The digest was dialysed and the dialysate hydrolysed for four hours with strong hydrochloric acid. The acid was removed and the hydrolysate electrodialysed. The neutral fraction from the electrodialysis was treated with sufficient charcoal to remove the aromatic amino-acids[5], and the residual neutral amino-acid mixture was then fractionated on a 'Zeokarb 215' column by the method of Partridge[6]. A fraction was obtained from the 'Zeokarb' column which produced a typical fit in a ferret when fed for three days at a level of 17 mgm. nitrogen per day. This active material contained at least fifteen components and was further fractionated by chromatography on a paper column ('Solka Floc') using a mixture of butanol, acetic acid and water as solvent. The toxic factor crystallized when the appropriate fraction of the effluent was concentrated.

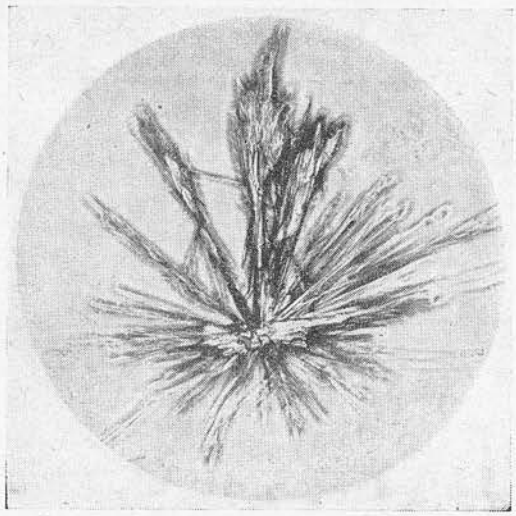
Toxicity

The crystalline toxic factor (see accompanying illustration) produced severe epileptiform fits in a ferret when fed for five days at a level of 100 μgm. nitrogen per day; this represents a total dose of crystalline material of approximately 3 mgm. The pure toxic factor is thus 33,000 times as toxic as the original flour.

When fed as a single dose the crystalline material is slightly more toxic than when the dose is spread over several days. A single dose of 2 mgm. is sufficient to produce typical epileptiform fits in a ferret, and a slightly greater dose kills the animal. Particular emphasis must be laid, however, on the cumulative nature of the poisonous effect. We have demonstrated in the ferret that a fatal toxic reaction can be produced by feeding the concentrated toxic factor in small doses over a period of five to ten days, and that the daily intake in such cases is only a fraction of the dose required to produce an immediate toxic response. It must be added, however, that this toxic action is not a simple problem and is probably influenced by other elements in the diet.

Nature of the Toxic Factor

As the amount of toxic factor in flour is small (1 gm. per 33,000 gm. flour) and the isolation procedure gives an overall yield of only 10 per cent, the greatest difficulty has been experienced in obtaining sufficient material for combined biological and chemical characterization. All the results reported below have been obtained on about 10 mgm. of crystalline toxic factor.



Crystalline toxic factor from agenized flour (× 180, phase contrast)

We were initially of the opinion that the toxic factor was a peptide, since a sample hydrolysed for twenty-four hours with 6*N* HCl at 110° and examined by partition chromatography[7] in two dimensions on paper was found to give six ninhydrin-positive spots. One of these spots was, however, identified as the toxic factor itself, and even forty-eight hours hydrolysis did not destroy it entirely. As a peptide could scarcely be expected to resist such vigorous hydrolytic conditions some doubt arose in our minds as to the peptide nature of our material.

A specimen of toxic factor hydrolysed for 24 hr. with 6*N* hydrochloric acid was studied in detail and the following degradation products were provisionally identified by paper chromatography : homocysteic acid, α-aminobutyric acid, methionine sulphoxide, methionine sulphone and homoserine. Since all these compounds can be regarded as possible degradation products of a molecule containing the methionine skeleton, we concluded that our toxic factor was probably derived from methionine. A small sample of the crystalline material was desulphurized by treatment with Raney nickel[8], and the product identified by paper chromatography as α-aminobutyric acid ; methionine gave α-aminobutyric acid under similar conditions.

It might be thought from the above evidence that the toxic factor was produced from methionine by a simple reaction with nitrogen trichloride. However, this would not appear to be so, for Silver[9] has shown

that when methionine is treated with nitrogen trichloride the toxic factor is not produced. We have recently treated the following peptides (kindly supplied by Dr. Fruton) with nitrogen trichloride: methionyl - glycine, carbobenzoxymethionyl - methionine amide and carbobenzoxymethionyl - methionine, and were unable to detect the presence of the toxic factor by partition chromatography after either a 4- or 16-hr. hydrolysis with strong hydrochloric acid at 110° C.

Comparison of Toxic Factors from Wheat Flour and Zein

Dr. Reiner, of Wallace Tiernan and Co., has recently kindly supplied us with a sample of a crystalline toxic factor which had been isolated from zein treated with nitrogen trichloride. Our crystalline material was indistinguishable from that of Dr. Reiner when examined by two-dimension partition chromatography on paper. The melting points of the two materials were within the same range, and both materials, fed to ferrets, produced the same toxic reaction.

While this work was in progress three communications[10] appeared from the laboratory of the British Flour-Millers Research Association, St. Albans, on a toxic factor present in nitrogen trichloride-treated zein. Although we have not been able to carry out a direct comparison between our own material and that isolated by this group, we find that the published details on the properties of the crystalline material isolated at St. Albans are in close agreement with our own. It seems highly probable, therefore, that the material isolated by us from agenized flour is identical with the material isolated by Dr. Reiner and independently by the St. Albans group from nitrogen trichloride-treated zein. We are indebted to Mr. J. Smiles for the photograph of the crystalline toxic factor and to Dr. C. Dent for samples of homoserine and α-aminobutyric acid.

[1] Mellanby, E., *Brit. Med. J.*, **2**, 885 (1946).

[2] Mellanby, E., *Brit. Med. J.*, **2**, 288 (1947).

[3] Newell, G. W., Erikson, T. C., Gilson, W. E., Gershoff, S. N., and Elvehjem, C. A., *J. Amer. Med. Assoc.*, **135**, 760 (1947).

[4] Radomski, J. L., Woodward, G., and Lehman, A. J., *J. Nut.*, **36**, 15 (1948).

[5] Tiselius, A., Drake, B., and Hagdahl, L., *Experientia*, **3**, 21 (1947).

[6] Partridge, S. M., *Biochem. J.*, **44**, 521 (1949).

[7] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).

[8] Fouken, G. S., and Mozingo, R., *J. Amer. Chem. Soc.*, **69**, 1212 (1947).

[9] Silver, M. L., Monahan, E. P., and Klein, J. R., *Proc. Soc. Exp. Biol.. N.Y.*, **66**, 410 (1947).

[10] Bentley, H. R., McDermott, E. E., Pace, J., Whitehead, J. K., and Moran, T., *Nature*, **163**, 675 (1949); **164**, 438 (1949); **165**, 150 (1950).

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The Isolation of a Toxic Substance from Agenized Wheat Flour

By P. N. CAMPBELL and T. S. WORK
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In 1937, Melnick & Cowgill reported the occurrence of 'running fits' in dogs when gliadin was the sole source of protein in the diet. Similar symptoms were not produced when gliadin was replaced by other proteins. The authors concluded that a toxic substance accumulated in the body when gliadin was fed. Wagner & Elvehjem (1944) examined the effect of adding various supplements to a gluten-rich diet and also concluded that 'running fits' were caused by a toxic substance in wheat products rather than by any nutritional deficiency.

In 1946, Mellanby showed that wheat flour which had been 'improved' by treatment with nitrogen trichloride (agenized flour) produced symptoms in dogs similar to running fits; a flour which had not been agenized was not toxic.

Much wheat is grown to-day which, if freshly milled, produces flour which is unsuitable for use in baking. If, however, such wheat is allowed to become aged or 'oxidized' by means of long periods of storage and is then milled, the resulting flour has greatly improved baking properties. This 'oxidizing' process can be more quickly brought about by the use of various oxidizing agents or 'improvers' on the flour. Nitrogen trichloride ('agene') was introduced as an artificial ageing agent by Baker (1921), hence the term agenized flour. The use of nitrogen trichloride has gradually spread, both in this country and in the United States, until in 1946 it was estimated that over 90% of the flour milled in England was treated with nitrogen trichloride. A review, in which the use of oxidizing agents in the treatment of flour is discussed, has been published by Blish (1945).

When agenized flour forms a substantial part of the diet of dogs, the animals become progressively more restless over a period of days and eventually develop epileptiform fits. If the diet is replaced at this stage by an exactly similar diet containing untreated flour the animals recover, but if the diet of agenized flour is continued the animals become progressively worse and die. Similar fits cannot be produced by unagenized flour (Mellanby, 1946) so that it must be assumed that the gliadin used by Melnick & Cowgill (1937) had been treated with agene.

There are striking differences in the susceptibility of different species to the toxic action of agenized flour. Mellanby (1947) showed that the ferret was nearly as sensitive as the dog, but that rats and mice did not develop fits. Newell, Erickson, Gilson, Gershoff & Elvehjem (1947) showed that guinea pigs and chicks were not susceptible, and Radomski, Woodard & Lehman (1948) showed that rabbits developed typical fits.

When the present investigation was begun it was already known (Mellanby, 1947) that the toxicity of agenized flour was associated with the gluten fraction, and that other proteins could also be rendered toxic by treatment with nitrogen trichloride (Moran, 1947). In view of the widespread use of agenized flour it became important to isolate and determine the nature of the toxic substance present in wheat flour. The present communication is concerned with the method used in the isolation of the substance and with its properties. A preliminary account of this work has already been published (Campbell, Work & Mellanby, 1950). The ferret has been used as the test animal and the toxic dose has been regarded as that quantity of material which would produce a typical epileptiform fit.

While the present work was in progress Bentley, McDermott, Pace, Whitehead & Moran (1949*b*) isolated from zein treated with nitrogen trichloride a crystalline substance which was toxic to rabbits. As indicated in the appropriate section this material appears to be identical with the material isolated by us from wheat flour.

MATERIALS AND METHODS

As an example of the methods used in the isolation of the toxic substance from agenized wheat flour, the treatment of a typical batch by the method finally adopted is described. Details showing the course of the isolation, the losses involved and the toxic dose at each stage are to be seen in Table 1. It should be emphasized that the figures quoted in this table are in some cases only an approximation; for although the losses in toxicity and the variation in the toxic dose which resulted from each step were determined, it was not possible to carry out each determination during the course of the fractionation of the same sample of wheat flour owing to the large quantities of material required for each

biological test and the lack of a micro-test. The figures in the table, therefore, represent a composite picture built up from results obtained during the course of several fractionations, but they do represent the course of the fractionation in a qualitative manner. It should be further emphasized that while we have every reason for confidence in the biological test employed, i.e. the production of an epileptiform fit in a ferret, no extensive experiments have been carried out to determine the mean toxic dose of any one preparation over a series of animals, so that the figures for the toxic dose quoted are again only an approximation. The fact that it has been necessary to carry out the biological test by the addition of the test substance to the normal food of the animal rather than by injection also contributed to the difficulties.

Separation of gluten from flour and treatment with pepsin and trypsin

Flour (17·7 kg.) which had been treated with 11 g. NCl_3 by Wallace and Tiernan Co. Ltd., Chiswick, London, was placed in a muslin bag in small batches and kneaded with running water until all the water-soluble material had been removed. The insoluble gluten was then mixed with 13 l. of distilled water containing 30 ml. of conc. H_2SO_4. Pepsin (40 g.) (pepsin powder, B.P., supplied by Chas. Zimmerman and Co. Ltd.) was added and the whole incubated at 37–38° with constant stirring for 24 hr. The pH of the suspension was then brought to 8·1–8·4 by the addition of 5N-NaOH and trypsin (40 g., supplied by British Drug Houses Ltd.) was added. A little toluene was added to the suspension which was incubated as before for 48 hr. The pH of the suspension, which tended to fall during the early part of the digestion, was checked every few hours and adjusted to 8·1–8·4 by the addition of 2N-NaOH. If the pH at any time fell below 7·0 it was assumed that the trypsin had been destroyed and a further 40 g. of trypsin were added. The suspension was then filtered and the residue washed with distilled water. The total volume of the digest and washings was about 14 l. which contained approximately 150 g. N and was equivalent to 101 toxic doses (fraction *A*, Table 1).

Preliminary experiments on enzymic digest

Before attempting to develop a method of fractionation, some general knowledge of the properties of the toxic substance had to be obtained.

Heat stability. A sample of digest was boiled for 1 hr. at pH 5·0 and the precipitated protein was removed; activity was not decreased.

Acid stability. Samples of digest were boiled with 6N-H_2SO_4 for 1, 4 and 18 hr. The SO_4^{--} was removed as $BaSO_4$ and each sample was biologically tested. Up to 4 hr. there was less than 20% loss in activity (a 20% loss was considered to be the minimum detectable without recourse to treatment of a group of animals and statistical analysis of the result). The sample heated for 18 hr. retained less than half of the activity of the digest.

Alkali stability. A sample of digest was heated for 1 hr. with excess 2N-$Ba(OH)_2$ and Ba^{++} removed as $BaSO_4$. No loss in activity could be detected.

Dialysis. A sample of digest was dialysed against successive portions of distilled water until no more material dialysed. The dialysate and residue possessed the same toxicity relative to their N content, but only 25% of the activity was retained in the dialysis sac.

Liberation of NH_2 groups by acid. The Van Slyke HNO_2 method was used to estimate the apparent NH_2-N:total N ratio. When the digest was boiled with 6N-H_2SO_4 for 4·5 hr. the proportion was increased from 27 to 54%, while boiling with conc. HCl for 3·5 hr. increased the ratio to 61%. Thus a greater degree of hydrolysis in a shorter time was effected with HCl than with H_2SO_4. The loss in activity after boiling for 3·5 hr. with conc. HCl was slightly greater than after boiling for 4·5 hr. with 6N-H_2SO_4, but the average molecular weight as indicated by the ratio NH_2:total N was smaller after HCl hydrolysis and this was accordingly adopted as a standard hydrolytic procedure.

Dialysis of enzymic digest

The enzymic digest (*A*, 14 l., 150 g. N) was concentrated under reduced pressure on a water bath (40–60°) to approximately 6 l. and was dialysed in cellophan sacs against 15 l. of distilled water at approximately 5°. Half the dialysate was removed after the first 2 days and replaced with an equal volume of distilled water. This process was repeated three times, after which the whole of the dialysate was replaced by distilled water three times. At the end of this time the amount of dialysable material which could be collected in 2 days did not represent more than 1% of the total N originally contained in the sacs. The combined dialysates were concentrated to 5·5 l. under reduced pressure at 40–60°. The total N in the combined concentrated dialysates was 125 g. (fraction *B*).

Acid hydrolysis of the dialysate

The dialysate (*B*, Table 1) was hydrolysed in four batches with conc. HCl by boiling 1·5 l. with 6 l. of 10N-HCl for 3·5 hr. under a reflux condenser. Most of the acid was subsequently removed by evaporation of the hydrolysate under reduced pressure on a water bath at 40°. The residual gum was diluted with a little water and reconcentrated to remove a further quantity of HCl. The combined filtered hydrolysates were diluted to 27 l. with distilled water and treated with the anion-exchange resin 'Deacidite E' (obtained from The Permutit Co.). The resin (approx. 2 kg.), after being well washed with distilled water, was activated by allowing it to stand overnight in 5 l. of 2N-NaOH, washed with 10 l. of distilled water by decantation, loaded into a porcelain pipe (11·5 × 75 cm.), and washed with distilled water until 100 ml. of effluent required less than 3 ml. of 0·02N-HNO_3 to neutralize it to methyl orange. The acid hydrolysate solution was added to the column and the effluent collected. When the pH of the effluent fell below 5·5 the column was washed clear of hydrolysate with distilled water and was regenerated with 0·5N-NaOH (30 l.) and washed as before with distilled water. The remaining acid hydrolysate was then added together with the acid effluent obtained from the first column. The combined effluents were concentrated under reduced pressure on a water bath to approx. 12 l. so that the concentration of N was about 10 mg. N/ml. (total N = 118 g., fraction *C*, Table 1).

Electrodialysis of acid hydrolysate

After decolorizing the neutral acid hydrolysate (*C*) by boiling with a little activated charcoal, the hydrolysates were subjected to electrodialysis after the method of Cox, King & Berg (1929). The apparatus consisted of a Perspex box divided into three sections each of which had a total

Table 1. *Stages in the isolation of toxic substance from wheat flour*

Process	Toxic dose	Total N available	No. of doses
Gluten	—	220 g.	—
Treated with 11 g. NCl_3	1·6 g. N	220 g.	140
After enzymic digestion (*A*)	1·5 g. N	Approx. 150 g.	101
After dialysis against distilled water (*B*)	1·5 g. N	125 g.	83
After hydrolysis with conc. HCl (*C*)	2·3 g. N	118 g.	50*
After electrodialysis	700 mg. N	35 g.	—
After removal of crystalline material (*D*)	500 mg. N	25 g.	50
After removal of aromatic amino-acids on charcoal (*E*)	460 mg. N	23 g.	—
After fractionation on Zeo-Karb†	—	—	—
Total of all active fractions	170 mg. N	5·5 g.	32
After removal of crystalline material	130 mg. N	4 g.	32
Most active fraction from column	96 mg. N	2·2 g.	23
After removal of crystalline material (*F*)	56 mg. N	1·3 g.	23
After fractionation on paper column‡ (*G*)	4 mg. N	64 mg.	16
Crystallization from aqueous ethanol	3 mg. solid	—	12

* Acid hydrolysis + electrodialysis caused a loss of one-third of the toxic substance. This is assumed to occur during acid hydrolysis during which stage there is little loss of N, thus the toxic dose goes up.

† For fractionation from Zeo-Karb column, see Table 3.

‡ For fractionation from paper column, see Table 4.

volume of 4 l. The most satisfactory membranes were prepared by treatment of animal parchment (plain skins from Witherby and Co., 326 High Holborn, London, W.C. 1) with 10% (w/v) formaldehyde for 5 hr. at 20°. These membranes had great mechanical strength and electro-endosmosis was minimal. The centre compartment of the box was fitted with a mechanical stirrer and the outer walls of the electrode compartments (platinum electrodes) were cooled (for a review of electrodialysis methods cf. Svensson, 1948). The hydrolysate (3–4 l.; 30–40 g. N) was poured into the centre compartment of the box and the end compartments were filled with distilled water. The electrodes were then connected to a 230 V., d.c. supply main with a 100 Ω variable resistance in the circuit. The current rose rapidly to 2·5–2·8 amp., the pH of the anode compartment fell and that of the cathode compartment rose. The duration of the dialysis depended on the amount of N initially added to the centre compartment and on the membranes, but for 40 g. N there was usually a fall in current to about 1·8 amp. in 24 hr. At the end of this time the contents of the end compartments were withdrawn (all but about 200 ml.), and were replaced with distilled water. Within the next 4–8 hr. the current fell to 0·4 amp. and then remained steady. At this point electrodialysis was considered to be complete.

Table 2. *Distribution of nitrogen during electrodialysis of acid hydrolysate*

(Results expressed as g. N.)

	Compartment		
	Cathode	Centre	Anode
Mean values for four runs	9·6	5·0	14·2
Percentage of total N	33	17	50

While the major part of the toxic substance remained in the centre compartment during dialysis a small amount travelled to the cathode compartment so that the contents of the latter were collected, concentrated under reduced pressure and re-electrodialysed together with sufficient of the anode solution to bring the pH to 5·5. The anode solution was not toxic. The total amount of N in the combined neutral fractions from the electrodialysis was 35 g., the toxic dose containing 700 mg. N. The combined neutral fractions were concentrated under reduced pressure on a water bath until crystallization took place. The crystalline material was removed by filtration and the concentration repeated until no further crystallization took place. By this means the total N in the solution was reduced to 25 g. and the toxic dose to the equivalent of 500 mg. N (fraction *D*, Table 1). The distribution of N during four electrodialyses is shown in Table 2.

Removal of aromatic amino-acids with charcoal

The aromatic amino-acids were removed by adsorption on activated charcoal. The charcoal was first treated with acetic acid according to the method of Tiselius, Drake & Hagdahl (1947) and then used as described by Schramm & Primosigh (1943). The filtrate (*D*) was treated in batches such that each batch contained approximately 4 g. N. Each batch was first diluted with 5% (v/v) acetic acid until the concentration of N was 1 mg. N/3 ml. Preliminary experiments on a pilot scale had shown that less than 20% of the N was in the form of aromatic amino-acids and this figure was used as a basis for the calculation of the amount of charcoal required; 1 g. of charcoal being required to adsorb 1 mg. of aromatic amino-acid N. The required amount of charcoal in a suspension in 5% acetic acid was poured into a Büchner funnel and washed with further quantities of 5% acetic acid. When the charcoal was nearly dry the solution of amino-acids was added and the effluent collected at a rate of approximately 5 l./hr. The charcoal was then washed with a volume of 5% acetic acid equivalent to 25 ml./g. of charcoal present, at a rate of approximately 10 l./hr. The effluent and washings were combined. The total amount of charcoal required to treat the 25 g. of N was 5 kg. and the total effluent amounted to 200 l. (fraction *E*).

Fractionation on Zeo-Karb 215 *column*

The effluent (*E*) from the charcoal was divided into three parts (approx. 10 g. N each) for fractionation (Partridge, 1949) on a cation-exchange column of Zeo-Karb 215 (obtain-

able from The Permutit Co.). The resin, after grinding, was sieved and 700 g. of the 60–80-mesh material was activated with HCl as described by Partridge & Westall (1949). The Zeo-Karb was poured into a column 5·8 cm. wide by 91·4 cm. long as an aqueous suspension and allowed to settle. After all the resin had been added, distilled water was forced up the column from the bottom so that the particles were freely suspended; the water flow was then reversed and the particles allowed to fall into position. By this means an evenly packed column is ensured. The column was washed with distilled water until the pH of the effluent had risen to 5·0–6·0.

The charcoal effluent (66 l.) was added to the column at such a rate that the outflow was approximately 5 l./hr. Ninhydrin tests were carried out on the effluent to check that the column was not being overloaded; the presence of a little glutamic acid in the effluent was ignored. When all the charcoal effluent had been added the amino-acids were displaced with 0·4 M-NH_3 solution added at a rate of approximately 1·5 l./hr. As soon as a significant amount of amino N appeared in the effluent it was collected in 20 ml. fractions by means of a fraction collector. A sample from every fifth tube was run on a one-dimensional paper chromatogram in butanol-water-acetic acid solvent (4:5:1) (Partridge, 1948) according to the method of Consden, Gordon & Martin (1944) in order that the amino-acid composition of the effluent might be followed. The elution with NH_3 was continued until the amount of N in the effluent became insignificant. The contents of the tubes were bulked into ten fractions according to their amino-acid composition. These were concentrated by evaporation under reduced pressure and N determinations (Kjeldahl) carried out. The distribution of N in a typical column effluent is shown in Table 3. Each of the last six fractions, in which proline was either absent or only present in very low concentration, was further concentrated and any crystalline material removed. The various fractions were then submitted to biological tests, the results of which are also shown in Table 3. It will be seen that although the activity was present in all of the last six fractions, it was concentrated in one fraction (*F*). The distribution of activity in the combined fractions from all three Zeo-Karb columns is shown in Table 1. It will be seen that for the most active fraction the toxic dose contained only 56 mg. N.

Before the Zeo-Karb was used again it was treated with 5% (w/v) NaOH solution at 55–60° to remove any aromatic amino-acids which may have been adsorbed and was then regenerated with 5 N-HCl (Newkirk & Handelman, 1949).

Fractionation of effluent from Zeo-Karb 215 on a paper column

The use of a column consisting of powdered paper was originally suggested by Consden *et al.* (1944). In the present case a powdered paper known commercially as solka floc (200-mesh grade; obtainable from Johnsen, Jorgensen and Wettre, 26 Farringdon Street, London, E.C. 4) has been used. The paper was washed thoroughly with distilled water, boiled for 30 min. with 5% acetic acid, washed with water, boiled with absolute ethanol, filtered, washed with ether, and dried in an oven at 110°. Approximately 1 kg. of the dried paper was then suspended in about 6 l. of acetone and loaded into a glass column 120 cm. long by 6·5 cm. diameter. The paper suspension was added in a continuous stream and the acetone allowed to flow freely from the column. After all the suspension had been added the column was washed with a further 6 l. of acetone. The level of the acetone was allowed to fall until it was just above the top of the paper and a solvent consisting of a mixture of butanol, water and acetic acid added. The composition of this solvent was similar to the 4:5:1 mixture described by Partridge (1948), but it was more economically prepared by mixing *n*-butanol 63%, water 27% and acetic acid 10% by volume together and allowing it to stand for 48 hr. before use. During this time esterification takes place and equilibrium is established; if the solvent separated into two layers then only the upper layer was used. The solvent (15 l.) was passed through the column at as fast a rate as possible until the paper had become saturated with water. This could be checked by adding a drop of water to 10 ml. samples of the effluent, since, when the solvent is saturated with water, a cloudy solution is produced under these conditions. The solvent level was then reduced to the level of the paper and the column was ready for fractionation.

Only the most active fractions (*F*, Table 1; 194–205, Table 3) from the Zeo-Karb column were fractionated on the paper column, the less active fractions being combined and first refractionated on a further Zeo-Karb 215 column. The solution of amino-acids to be fractionated (300 mg. N) was evaporated to dryness *in vacuo*, a little ethanol added and the evaporation repeated twice. The residue was dissolved in about 300 ml. of the butanol-acetic acid solvent to which 6 ml. of glacial acetic acid and 10 ml. of ethanol had been added. This usually resulted in a quite clear solution, but in the event of the solution being cloudy it was first cleared by centrifugation and only the supernatant solution used for fractionation. The clear solution was then added to the

Table 3. *Fractionation on Zeo-Karb* 215

Fraction tubes no.	N from column (mg.)	N after removal of crystalline material (mg.)	No. of toxic doses	N/toxic dose (mg.)
76–123	1930			
124–159	1700	—	Not toxic	Not toxic
160–171	547			
172–188	700			
189–193	216	162	1	162
194–205	618	375	8	47
206–213	245	173	1·5	115
214–220	212			
221–239	174	183	1	183
240–261	18			
Total N in mg.	6360	893	—	—

column without disturbing the surface. This solution was allowed to percolate into the paper before the developing solvent was added. The flow rate was adjusted to 1 drop/sec. and the effluent was collected in 18 ml. fractions on a fraction collector, approximately eighty fractions being collected in 24 hr. Samples from every fifth tube were run on single-dimensional paper chromatograms (Consden *et al.* 1944) in butanol-acetic acid in order to determine the amino-acid composition of the effluent. The fractions were then grouped according to the composition of their ninhydrin-positive substances as shown in Table 4. All the groups were biologically tested, but only one (*G*, Table 1, 355–475, Table 4) possessed any activity; a typical fit being produced by material containing as little as 4 mg. N. The ninhydrin-reacting substance in (*G*) gave initially a yellow colour with ninhydrin, but on heating the colour changed through brown to the usual purple.

Table 4. *Fractionation on paper column*

(300 mg. N on 1 kg. paper. Each tube contained 18 ml. of solvent.)

Tube no.	Total N (mg.)	Amino-acid composition
150–180	68	Leucine + trace valine
181–209	53	Valine + trace leucine
210–229	22	Unidentified, purple ninhydrin
230–255	56	Proline
256–269	18	γ-Aminobutyric acid
270–282	6·5	Traces of various unidentified ninhydrin positive substances
283–304	25	
305–315	8·2	
316–340	14·6	
341–354	5·6	
355–475	16	Toxic substance
476–		Histidine positive Pauly reaction

When the amount of ninhydrin-positive material in the fractions fell to an insignificant amount the solvent was changed to an 85% ethanol-water mixture in order to remove the last traces of substances with a very low R_F. 2 l. of 85% (v/v) ethanol followed by 6 l. of 50% (v/v) ethanol were added and the effluent collected. After concentration under reduced pressure these fractions were tested biologically, but failed to give any positive reaction. The solvents were then used in the reverse order. After the addition of 2 l. of butanol-acetic acid solvent the column was again ready for further fractionations.

Treatment of the active fraction from the paper column

The fractions (*G*, Table 1; 355–475, Table 4) from the paper column were pooled and evaporated to dryness under reduced pressure. The acetic acid was removed by the addition of successive small quantities of distilled water and evaporation to dryness. The residue was dissolved in the minimum amount of distilled water and any insoluble material was filtered off. After the addition of a little absolute ethanol to the solution the toxic substance crystallized in the form of small colourless beads. The toxic dose of this crystalline material, m.p. 226–232° (decomp.), when fed to a ferret over a period of 5 days was 3 mg. of solid. The total number of toxic doses obtained from 17·7 kg. of flour was about 12, giving an overall yield of 8·5%. A phase-contrast photograph of the recrystallized material is shown in Fig. 1.

Alternative methods of isolation

While this work was in progress, Bentley, McDermott, Pace, Whitehead & Moran (1949*a*) reported the purification of a toxic substance from agenized zein. In many respects the methods used by these workers corresponded to those employed in the present work. It seemed possible that some of their methods might also prove useful in the extraction of a toxic substance from flour.

Butanol extraction. A sample of an active fraction from a Zeo-Karb 215 column equivalent to 380 mg. N (two toxic doses) was made up to 50 ml. with distilled water and extracted with *n*-butanol at 60° for 22 hr. as described by Dakin (1918). On examination of the two extracts by paper-partition chromatography in butanol-acetic acid-water it was found that the butanol fraction contained most of the amino-acids (357 mg. N). The water fraction was completely lacking in valine and leucine and contained 21·5 mg. N. Biological tests showed that both fractions were of approximately equal activity. Although this method was attractive in that the total N for one toxic dose was reduced from 190 to 21·5 mg. N the loss of 50% of the toxic material rendered the process uneconomic. The appearance of 50% of the activity in the butanol extract was contrary to the experience of Bentley *et al.* (1949*a*). This discrepancy must now be attributed to some small difference in technique as it appears that the toxic substance from wheat flour is identical with that from zein.

Partition between phenol and acid. A sample of the concentrated toxic factor from Zeo-Karb 215 was partitioned between equal volumes of phenol and 0·1 N-HCl. The amino-acids were distributed almost equally between the two layers and the method was not considered useful in the present case.

Degradation of crystalline toxic substance

As the quantity of crystalline material available after biological testing was about 10 mg. it was not possible to isolate and characterize degradation products. When the identity of a degradation product had been indicated by chromatography on paper it was mixed with an authentic specimen and an attempt made to separate the substances by partition chromatography in two dimensions on paper (phenol/NH_3 followed by acetic acid/butanol/water). If no separation was achieved the unknown was assumed to be identical with the known specimen.

With this reservation, the following substances were identified as the products of acid hydrolysis (6 N-HCl for 24 hr.) followed by treatment with H_2O_2; homocysteic acid, methionine sulphoxide, methionine sulphone, α-aminobutyric acid and homoserine. In addition, a small quantity of undegraded toxic substance was identified. The relative positions of these substances on a chromatogram is shown in Fig. 2.

A sample (2 mg.) of crystalline toxic substance was desulphurized by treatment with Raney nickel by the method of Fonken & Mozingo (1947). After removal of the solid nickel a small quantity of NiS was precipitated when H_2S was passed into the filtrate. After removal of NiS the filtrate was divided into four portions. One quarter was chromatographed on paper using collidine/lutidine as solvent. The R_F indicated that the degradation product was an aminobutyric acid. One quarter was now mixed with synthetic α-aminobutyric acid and one quarter with γ-aminobutyric acid. Each mixture was chromatographed on paper. The

mixture of unknown substance and γ-aminobutyric acid separated into two ninhydrin-positive spots; the mixture of unknown and α-aminobutyric acid could not be separated. Methionine gave α-aminobutyric acid under similar conditions.

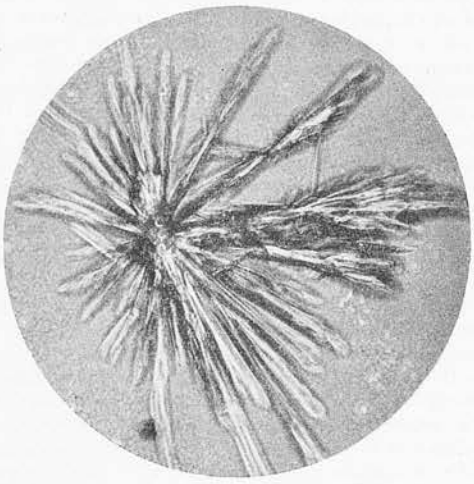

Fig. 1. Phase-contrast photograph of the crystalline toxic substance from agenized wheat flour (× 180).

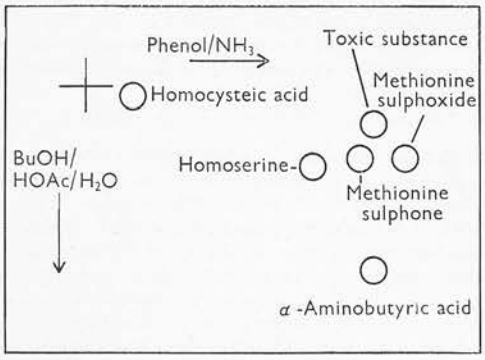

Fig. 2. Diagrammatic representation of the position of the toxic substance and its degradation products (acid hydrolysis) in a two-dimensional chromatogram.

Treatment of some methionine peptides with NCl_3

The following peptides were treated with NCl_3 in $CHCl_3$: methionylglycine, carbobenzyloxymethionylmethionine amide, methionylmethionine, carbobenzyloxymethionylmethionine, carbobenzyloxy-L-methionine amide, carbobenzyloxyglycyl-L-methionine and carbobenzyloxy-L-methionylglycine. A sample (20 mg.) of each was suspended in approximately 10–15 ml. of $CHCl_3$. Air containing NCl_3 from approximately 50 ml. of Cl_2 was passed into each. The solutions were left at room temperature for about 1 hr. when the remaining NCl_3 was removed by sucking a vigorous stream of air through the solutions until no further $CHCl_3$ remained. Each specimen was dissolved in 6 N-HCl (0·2–0·3 ml.) and heated in a sealed tube for 11 hr. at 100–105°. The amino-acid composition of each hydrolysate was studied by two-dimensional paper-partition chromatography using butanol acetic acid and phenol/NH_3 as solvents. Wherever a component was detected which resembled the toxic factor in R_F values a second chromatogram was run with a mixture of peptide hydrolysate and crystalline toxic substance. In every case it was possible to separate the toxic substance from all the peptide degradation products and it was, therefore, concluded that in no case had the NCl_3 treatment of a peptide given rise to the toxic substance.

RESULTS AND DISCUSSION

Isolation

In order to isolate the toxic substance from wheat flour it was obviously desirable to obtain a flour possessing the maximum activity. As a result of many experiments it was concluded that it was not practicable to treat wheat flour with more than about ten times the amount of nitrogen trichloride which was normally used commercially. In the later stages of the work commercial gluten itself was treated with nitrogen trichloride, since the toxic substance is associated with the gluten fraction of the flour, but the product was only slightly more active than that obtained when the flour itself was treated with nitrogen trichloride. The digestion of agenized gluten with pepsin and trypsin resulted in a very considerable reduction of the average molecular weight, for about 80 % of the nitrogen was dialysable at this stage. Since on a nitrogen basis the non-dialysable material was as toxic as the dialysable, it was apparent that the toxic substance was an integral part of the protein and was not a low-molecular-weight impurity.

Although it is possible that the non-dialysable residue from the enzymic digestion could have been satisfactorily hydrolysed by acid, it was felt that the elimination of possibly 'resistant' non-dialysable peptides at this stage was an advantage.

The acid hydrolysis caused a destruction of about 30 % of the toxic substance (see Table 1), but the proportion of amino nitrogen to total nitrogen was raised from 25 % to approximately 60 %; thus a further substantial reduction in the average molecular weight was achieved. It was felt that fractionation would be so simplified by this reduction in molecular weight as to make an acid hydrolysis an essential step in spite of the ensuing loss of activity.

Electrodialysis achieved a very considerable purification of the toxic substance with negligible loss. Table 1 shows that the toxic dose contained 2300 mg. nitrogen before electrodialysis and 500 mg. nitrogen after electrodialysis and removal of crystalline material from the neutral dialysate. About 36 % of the total amino-acids in hydrolysed gluten consist of glutamic acid. Thus most of the nitrogen in the anode compartment was glutamic acid. The basic amino-acids, arginine, lysine and

histidine, together account for about 10% of the total solids and, of course, a very much higher percentage of the N. Thus it is not surprising that even though only a crude fractionation was achieved by this process a great reduction in the ratio nitrogen/toxic dose was achieved.

The removal of aromatic amino-acids by charcoal was found to be a necessary step before fractionation of the mixture on a Zeo-Karb column, since we, like Partridge (1949), found that in the presence of aromatic amino-acids fractionation on Zeo-Karb 215 is adversely affected.

The toxic substance was found in the fractions from the Zeo-Karb column immediately following proline. Although activity was spread over rather a large number of fractions, the maximum activity appeared in a relatively small fraction. Only this fraction was used for the next step in purification and the other active fractions were combined and refractionated on Zeo-Karb.

Single-dimension paper chromatography of the various active fractions from Zeo-Karb indicated the presence of at least two major abnormal components. One of these, eventually identified as the toxic substance, had R_F in butanol/acetic acid of 0·07 and in phenol/ammonia of 0·64. The second abnormal component was provisionally identified, in the first instance by paper chromatography, as γ-aminobutyric acid; it was then isolated, analysed and found indistinguishable from an authentic specimen of γ-aminobutyric acid. A yield of 46·7 mg. of thrice recrystallized γ-aminobutyric acid, m.p. 193°, was obtained from 1800 g. of gluten.

Since it has not been possible to isolate γ-aminobutyric acid from unagenized gluten by the methods which were successful in the case of agenized gluten, it must be concluded that γ-aminobutyric acid arises as a result of the action of nitrogen trichloride on the protein. Since all fractions from the paper column other than the one containing the toxic substance were biologically inactive, it is concluded that the toxicity of agenized wheat flour cannot be attributed in any way to the presence of γ-aminobutyric acid. Further, Keil (1932) did not detect any toxic action arising from the subcutaneous injection of the sodium salt of γ-aminobutyric acid to human subjects.

The most active fraction from Zeo-Karb was still a complex mixture, but a considerable quantity of neutral amino-acid was removed by concentration and crystallization. The activity remained entirely in the mother liquors and these were found, by two-dimension paper chromatography, to contain at least sixteen ninhydrin-positive substances.

Fractionation on a column of powdered paper was a most efficient method. The degree of fractionation depended on the amount of material put on the column. When a column containing 1 kg. of paper was loaded with a mixture containing 100 mg. nitrogen, fractionation was complete and every substance appeared in the effluent unmixed with substances of similar R_F values. When the load was increased to 300 mg. nitrogen the substances with high R_F values were not completely separated; when the load was increased to 500 mg. nitrogen even the components with low R_F values were mixed. The progress of fractionation of a 300 mg. nitrogen batch on a paper column is shown in Table 4.

In assessing the value of a paper column as a method of fractionation of a particular amino-acid mixture, it was of great convenience to find that a column and a paper strip behaved in a similar fashion. There were, however, small but significant differences, thus proline appeared in the effluent from the column before γ-aminobutyric acid, whereas on a paper strip, in the same solvent mixture, the order was reversed. The loss of about 30% of the toxic substance on the paper column cannot be explained.

Some difficulty was experienced in the crystallization of the toxic substance from the paper column. There was contamination with a small quantity of carbohydrate, presumably the product of slow decomposition of the very large quantity of paper in contact with acetic acid. Similar difficulties have been reported by Moore & Stein (1949) when using starch columns with acidic solvents.

Degradation and comparison with other toxic factors

With the reservation made in the appropriate experimental section, the identification of α-aminobutyric acid as a product of desulphurization with nickel suggested that the toxic substance was derived from methionine. The production of methionine sulphoxide and methionine sulphone on acid hydrolysis supported this view. The occurrence of homoserine, α-aminobutyric acid and homocysteine as products of acid hydrolysis suggested in the first instance that we were dealing with a peptide, but the failure to identify any product of desulphurization other than α-aminobutyric acid was contrary to this conclusion.

In the earlier stages of this investigation we were of the opinion that the toxic substance was a peptide and we believed that our substance differed from that which was produced from zein by treatment with nitrogen trichloride. This belief was occasioned by the suggestion of Bentley *et al.* (1949*a*) that a toxic factor which they had obtained in a highly concentrated form from zein did not give a reaction with ninhydrin. However, when Bentley *et al.* (1949*b*) crystallized their toxic substance from zein and degraded it to α-aminobutyric acid by hydrogenolysis (Bentley, McDermott, Pace, Whitehead & Moran, 1950) it became obvious to us that the toxic substance which we had isolated from wheat flour

was closely similar to and probably identical with that already isolated from zein. Identity was confirmed when Dr L. Reiner supplied us with a sample of the crystalline substance isolated from zein in a parallel, but independent investigation (Reiner, Misani, Fair, Weiss & Cordasco, 1950). Dr Reiner's material could not be separated from our own by two-dimension partition chromatography on paper, and on acid hydrolysis it gave rise to the same five ninhydrin-positive substances.

We have been unable, through lack of sufficient material, to obtain a full elementary analysis of our material; an analysis has, however, been published by Bentley *et al.* (1950) and the deduction has been drawn by these authors that the molecule $C_5H_{12}O_3N_2S$ can be regarded as being derived from methionine sulphoxide by addition of NH or from methionine sulphone by replacement of O by NH. All the hydrolytic degradation products which we have 'identified' by chromatography could be envisaged as possible degradation products of such a structure.

It seemed reasonable to expect that treatment of a single methionine peptide with nitrogen trichloride would give rise to the toxic substance, but, as indicated in the experimental section, we were unable to detect any formation of the toxic substance in the reaction between nitrogen trichloride and six different methionine peptides. It may be that the chromatographic method used to detect formation of the toxic substance was insufficiently sensitive and that a very low yield of toxic substance was obtained; it seems more probable, however, that the reaction conditions were not analogous to those which exist in the agenization of flour.

Although it has not been possible to produce typical epileptiform fits in all animals by feeding agenized flour, it cannot be assumed that the toxic factor is harmless in those animals which show no nervous response. In view of the very serious and eventually fatal outcome of the treatment of dogs, ferrets, rabbits and cats with the toxic substance, the use of nitrogen trichloride as an ageing accelerator in the manufacture of flour seems highly undesirable.

SUMMARY

1. The isolation of a toxic substance from agenized wheat flour is described.

2. The probable identity of this substance with a substance having similar physiological properties from agenized zein is indicated.

3. The toxic substance was isolated from an acid hydrolysate of the gluten fraction of flour and is regarded as part of a flour protein.

4. Large-scale partition chromatography on paper-pulp columns was developed as a method of fractionation of complex mixtures.

5. Degradation experiments suggested that the toxic substance was a derivative of methionine. The probable structure is given.

6. Methionine peptides treated with nitrogen trichloride did not give rise to the toxic substance.

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